ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

# Genetic Analysis Of Highly Pathogenic Avian Influenza H5N1 Sub Lineages Co-Circulating In Backyard Chicken In Egypt.

**Abd El Hafez Samir[1]*, Abd El Satar Arafa[1], Amany Adel [1]* Ahmed Abd EL Halim[1]*, Hesham A Sultan[2], and Hussein A Hussein[2]**

[1]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza (AHRI), Egypt.
[2]Faculty of veterinary medicine-Sadat university and Cairo university, Egypt.

**ABSTRACT**

Avian influenza viruses became widely distributed in most Middle Eastern countries, causing great economic losses in poultry industry especially when complicated with other pathogens. In the present study we molecularly characterize 10 H5N1 form chicken backyard in 2015, phylogenic analysis for Five genes (HA,PB1,PB2,PA,NS) of H5N1 viruses give genetic prospective of the virus circulating in Egypt, identifying specific mutations associated with antiviral sensitivity and enhance replication and virulence in mammalian species **,** this study indicate that the highly pathogenic avian influenza viruses circulating in Egypt in period from 2015 are belonging to clade 2.2.1.2 that related to 2006, all strains under study show presence of multiple basic amino acids at the HA cleavage site that considered the primary virulence factor .

**Keywords:** H5N1, avian influenza, virus, pathogens.

***Corresponding author***

ISSN: 0975-8585

## INTRODUCTION

Avian influenza is further classified into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), depending on the severity of the disease in susceptible birds. HPAI outbreaks in chickens and turkeys have been caused mainly by the H5 and H7 subtypes, however some of the H5 and H7 subtypes have been characterized as HPAI and many strains of these subtypes have been shown to be LPAI (Zhou et al., 1999). H5 and H7 strains remove ability to undergo mutation from LPAIV to HPAIV during replication and circulation in poultry (Spackman et al., 2002). Influenza type A has been isolated from different species including birds, Humans, pigs, horses, and sea mammals (Webster et al., 1992)..

The influenza viruses are medium-sized, enveloped, negative sense ssRNA viruses with a segmented genome. Taxonomically, they belong to the virus familyOrthomyxoviridae. There are three genetically and antigenically distinct types of influenza viruses called A, B, and C **(Cox et al., 2000)**. Type A viruses are further divided into subtypes according to the combination of two main envelope glycoproteins the hemagglutinin (HA) and neuraminidase (NA). To date, 17 HA subtypes (H1–H17) and 9 NA subtypes (N1–N9) have been found and almost all subtype combinations**(Adwan, 2009).**

Infection of domestic poultry by avian influenza (AI) viruses typically produces syndromes ranging from a symptomatic infection of respiratory disease and drops in egg production to severe systemic disease with near 100% mortality (Swayne and Halvorson, 2003).

Egypt experienced the disease since the first introduction of Highly Pathogenic Avian Influenza HPAI H5N1 in 2006(Aly et al., 2006).. The virus widely extended in very short time and infected commercial poultry production sectors and backyards (Aly et al., 2006-a, b). The virus is currently endemic among backyard poultry flocks in many provinces in Egypt

**The aim of this study are:**

1-collection of suspected cases o
f AIV subtypes H5 from chicken backyard
2- detection of the virus H5 by real time PCR
3-Isolation of the selected positive H5N1 samples from chicken backyard and provinces in SPF chicken eggs for virus propagation then confirmation by HA assay
5-Sequencing of whole HA gene of the tested isolates (H5N1) and study of genetic changes among the Egyptian viruses.
6- Sequencing of internal genes of the tested isolates (H5N1,),(NS, PA,PB1,PB2) and study of genetic genetic determinants
7- detection of genetic reasortment.

## MATERIAL AND METHODS

### Samples

Pooled oropharyngeal swabs were collected from 10 backyard chickens populations from 4governorates in Egypt (Table S1). Samples were collected during 2015. All the chicken backyard flocks suffered from respiratory disorders, nasal and ocular discharge. All the suspected samples were collected, isolated and examined in the Reference Laboratory of Veterinary Quality Control on Poultry Production (RLQP) in Egypt.

### RNA extraction and molecular diagnosis

Viral RNA was extracted from the samples using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were tested using one step RT-qPCR(Qiagen, Hilden, Germany) for the M gene of influenza type A viruses(Spackman et al., 2002)using the real-time PCR Mx3000P QPCR System (Agilent, California, USA). Positive AIV RNA was subtyped for HA using specific primers for the suspected H5subtype (Shabat et al., 2010), Thermal profile for amplification of HA gene of H5 subtype was done as follows: 50 °C for 30 min, 95 °C for 15 min, cycling steps of 94 °C for 10 s, 54 °C for 30 s and 72 °C for 10 s repeated for 40 cycles.

**Virus isolation**

Virus isolation and propagation was carried out on 9–11 day old SPFECE through intra-allantoic inoculation, then incubated at 37 °C for 3–5 days. The harvested allantoic fluids were tested for virus hemagglutination activity by HA assay (OIE, 2015). Collected allantoic fluid wastested by hemaglutination assay and specific RT-qPCRs

**Amplification of genes of H5N1 viruses:**

Total of 10 isolates were amplified and sequenced for Hemagglutinin gene, in addition to the genes that related with the pathogenicity (PB2, PB1, PA and NS) of the viruses were sequenced for 5 selected viruses were amplified by RT-PCR using Qiagen®kit (QIAGEN, Hilden, Germany) with primers described in (table S2). The RT-PCR was carried out as follows: one cycle of RT step at 50 °C for 30 min, then one cycle at 95°C for 15 min for initial denaturation followed by 40 cycles of 94°Cfor 45 s, 56°C for 45 s and 72°C for 2 min and final extension at 72 °C for 10 min. The test was carried out on thermocycler 2720 ABI (Applied Biosystems, USA). The electrophoresis of PCR products was done on Ethidium bromide stained agarose gel 1.5% and the amplified products were visualized by gel documentation system – Image capture (Biometra, Germany).

**Genes equencing:**

The amplicons for the different genes were purified then sequenced by Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA), using primer specific for HA gene as previously shown in supplementary Table 2. The cycling protocol for sequence reactions was done as follows: one cycle at 96 °C for 1 min, 25 repeated cycles of 96 °C for 10 Sec, 50 °C for 5 Sec and 60 °C for 2 min. Then, the sequencing reactions were purified using a spin column Centrisep® kit (Applied Biosystems, USA) to remove the extra free dNTPs bases, and followed by loading the purified reactions in a sequencer plate of ABI (Applied Biosystems 3130 genetic analyzers, USA).Sequences generated in this study were submitted tothe Genebank of the national center of biotechnology (NCBI) under accession numbers that were mentioned in table S3.

**Genetic and phylogenetic characterization:**

A BLAST search was performed using NCBI, and sequences used in this study have been retrieved from the NCBI database for representative H5N1 and other similar viruses. Alignment and identity matrix analyses were done using Bioedit software (version 7.1) **(Hall, 1999)**

The phylogenetic analysis was done by using maximum likelihood (ML) tree method by Mega6 software **(Tamura et al., 2013)**, we used the general time-reversible (GTR) model of nucleotide substitution with gamma-distributedrate variation among sites and estimated proportion of invariant sites (I) (with 8 rate categories, Γ4), with 1000 replicates of bootstrap analysis.The determination of glycosylation sites on hemagglutinin gene was accomplished by NetNGlyc 1.0 Server **(Gupta and Brunak, 2001).**

**RESULTS**

**Quantitative Real time PCR for H5 detection:**

By real time PCR, the 10 isolates were positive for H5 subtype with ct values ranged from 13.22 to 20.22 as listed in table S1. The positive samples were isolated and then examined for HA activity and the collected allantoic fluids have HA unites between 2log7-2log9

**Genetic analysis of the sequenced genes:**

**HA:**

All 10 isolates were H5 subtype highly pathogenic due to the presence of multiple basic amino acids in the cleavage site through sequence of full HA gene. Viruses collected in Egypt during 2015 showed some

variation in comparison with the original strain isolated in Egypt in early 2006 and also the viruses from Qinghai-lake and viruses from neighboring Middle Eastern and European countries (clade 2.2)

The Phylogenetic analysis of HA gene for chicken isolates (Fig. 1) and (Fig. 15,16) either based on nucleotide sequence or amino acid sequence show 10 isolates were grouped together and belong to clade 2.2.1.2 .(Arafa,etal2016)

The cleavage site sequence of tested isolates indicates all isolates have multiple basic acids in cleavage site and show one pattern present in all 10 isolates PQGEKRRKKRGLF.

Our study revealed that presence of mutations in antigenic epitopes of 10 isolates ranged between 4 to 26.
The only mutation in receptor binding site is 129 deletion which present in 10 isolates .

A phylogenetic tree of publicly available H5 HA sequences showed that more than 70% of Egyptian isolates lack the HA154–156 glycosylation site in comparison with Vietnam only 25% and Indonesia 0%. (i.e., countries with appreciable numbers of human H5N1 infections) revealed that Human H5N1 infections in Vietnam and Indonesia from 2009 to 2011 were mostly (Vietnam) or exclusively (Indonesia) caused by viruses with the HA154–156 glycosylation site. Phylogenetic analysis of all strains under study detect that all the group of subclade 2.2.1 lack the glycosylation site, this finding might suggest that avian H5N1 viruses lacking the HA154–156 glycosylation site transmit to humans more readily than those that possess the glycosylation site, at least in the genetic background ofEgyptian H5N1 viruses.( Neumann G et al.,2012).

**PB2:**

several studies indicated thatthe PB2 gene E627K , A199S,D701N and 271 are of molecular determinant of H5N1 virus pathogenicity and virulence in mice and polymerase activity in mammalian host cell(Hatta etal.,2001),(Bussey et al 2010),theses mutations weren't present in the current study **.**

In this study we are found: (I71M, R85K)(A111T, N134T, R202K, K254E, M297I,I220M,K274R except no 5 contain K294R,V534T,I575M,T666M)

**PB1**:

In this study make partial analysis of pb1 and presence in one samples only mutation T672I.

The Identity Percent for the Amino Acid sequence of whole PB1 gene of the 10 isolates in comparison with A/chicken/Egypt/06553/2006(H5N1) ranging between 99% to 100 %

**PA**:

It was found that PA gene of all strains of subclade 2.2.1 (Egypt/2.2.1) containN341S, S388R, A448E and K615R. K615R represent one of important potential markers of host adaptation(Gabriel et al 2005).

In the current study we found (S63G, I66T, V68I, V99I, N89S , I206V, K209R, R217C, N232K, K274K, S292A,
G326N,T342A,M347L,D356E,K366R,R372K,R393S,K396R,E404K,T406P,E353A,I509L,K541R,E549R,A558E,V559I,L690P,L591F,R620K,R631K,S668P,A674V,L712F,A717Tand N721R) .

The Identity Percent for the Amino Acid sequence of whole PA gene of the 10 isolates in comparison with A/goose/Guangdong/1/1996 )A/bar-headed/Qinghai/3/2005) A/chicken/Israel/625/2006\ (H5N1) ranging between 96% to 100%.

**NS:**

NS1 protein of Egyptian strains can be classified into 3 groups could be named A, B & C , each group has characteristic markers differ in between the groups as group (A) contain 44R , 48N and ESKV, group (B) contain

44K ,48N and ESKV while group (C)contain 44K,48S and ESEV.(Adel .A ,etal 2012). it was found that Isolates under study are closely related to group C.

In our study there are some mutations as:

(K49R,S53N,K60E,S181N,K76E,A81E,T165A,V203I,S221P,A230R,S234E).

The sample NO,2 A/chicken/Egypt/ABD2/2015 contain another new mutations D263E,A61T,G229R .and sample NO 5A/chicken/Egypt/ABD5/2015 contain two mutation as I190VandF175S.

The amino acid at position 42 of NS1 varies among avian influenza viruses; however, the serine (S) at this position is highly conserved in the human, swine, and equine influenza viruses. Based on the available sequence information, H5N1 influenza viruses that have been isolated from humans and other mammals have serine at position 42 in the NS1 protein. The amino acid at position 42 is located within the second α-helix spanning amino acids 30 to 50 of the RNA-binding domain of NS1 protein (Wang et al ,1999 ).

The NS1 gene is critical for the pathogenicity of avian influenza virus in chickens, and that the amino acid residue Ala149 (A) correlates with the ability of these viruses to antagonize interferon induction. the NS1 gene of GS/GD/1/96 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(Li, Z. et al, 2006) but a A/goose/Guangdong/2/96 (GS/GD/2/96) virus encoding a Val (V) 149 substitution is not capable of the same effect.

The Identity Percent for the Amino Acid sequence of NS gene of the 10 isolates in comparison with A/chicken/Egypt/06553/2006 and A/bar-headed/Qinghai/3/2005 ranging between 95 % to 100 % and with the virus strain of A/Goose/Guangdong/1/96(H5N1) ranging between 67.8 % and 69.3 %.

**DISCUSSION**

All 10 isolates were H5 subtype highly pathogenic due to the presence of multiple basic amino acids in the cleavage site through sequence of full HA gene. Viruses collected in Egypt during 2015 showed some variation in comparison with the original strain isolated in Egypt in early 2006 and also the viruses from Qinghai-lake and viruses from neighboring Middle Eastern and European countries (clade 2.2). Persistent circulation of HPAI H5N1 in Egypt has resulted in the emergence of a distinct sub lineage of H5N1 viruses within clad 2.2; termed clade 2.2.1.2 (, Balish et al., 2009,Arafa et al., 2010-b). The Phylogenetic analysis of HA gene for chicken isolates and (Fig. 1,2) either based on nucleotide sequence or amino acid sequence show 10 isolates were grouped together and belong to clade 2.2.1.2 (Arafa,etal2016)..

Amino acid identities ranged between 95%-100% among the analyzed isolates The isolates shared 95%-96%, 95%-99% and 92%-96% identity in the H5 gene amino acid sequences with isolates (A-duck-Egypt-2253-3-2006), (A-goose-Guangdong-1-96) (A-chicken-Egypt-06553-2006) and (A-chicken-Egypt-0880-2008) ), which is the original isolate isolated in early 2006 and other reference samples from the world.

The cleavage site sequence of tested isolates indicates all isolates have multiple basic acids in cleavage site and show one pattern present in all 10 isolates PQGEKRRKKRGLF.

Presence of multiple basic amino acids at the HA cleavage site is well established as an accurate indicator of virulence or potential virulence for H5 and H7 influenza viruses (Hoffmann et al., 2007).

Our study revealed that presence of mutations in antigenic epitopes of 10 isolates ranged between 4 to 26.

The only mutation in receptor binding site is 129 deletions which present in 10 isolates and also present in human influenza viruses H5N1, H3N2 and H1N1, an increasing prevalence of Δ129 HPAIV H5N1 viruses was also observed in sequenced isolates from poultry in Egypt. (Abdelwahab, etal, 2010)

In our study, the HA molecules of the Egyptian H5N1 strains retained the 2,3-NeuAcGal linkage properties, which share the same amino acids Q226 and G228 as avian viruses. The multiple basic amino acids at the HA cleavage site are essential for lethal infection in chicken and mouse, these results are consistent with the observations that viral isolate is highly pathogenic to chickens.

Several studies previously showed that Some mutations such as ( Q222L ,G224S, S223N,N182K,Q192R ,A134V) play a role in enhancing the binding of H5N1 Virus to the silica acid (SA) a2,6-Gal receptor (Zhu et al 2008 ).These Mutations were not found in the HA of Egyptian H5N1 strains under study , Implying that the isolates preferentially bind to a2,3-NeuAcGal Linkages of the avian cell receptors rather than a2,6-NeuAcGal Linkages of the human cell receptors .

the PB2,PB1 and PA subunits are tightly associated with each other to form a compact structure .there is binding between N terminus of PB2 (1-37 AA ) with C terminus of PB1 (678-757 )(Poole etal,2007) Forming very highly conserved region among all influenza Viral strains , PB1–PB2 interface appears as target for novel anti-influenza drugs of use against all strains of influenza A virus . Two important host specific markers in the pandemic isolates the PB2 gene E627K and A199S in the PB2 gene, which is well-known to be associated with increased virulence of A/H5N1 viruses for mice (Hatta etal.,2001) these mutation not present in current study. also There is another position D701N that considered one of molecular determinant of H5N1 virus pathogen city in mice and not recorded in all Egyptian strains from 2006 , PB2 residue 271 plays a role in the enhanced polymerase activity of influenza A viruses in mammalian host cells(Bussed et al 2010),This study not contain these mutation.

In this study we are found: (I71M, R85K)(A111T, N134T, R202K, K254E, M297I,I220M,K274R except no 5A/chicken/Egypt/ABD5/2015contain K294R,V534T,I575M,T666M) the importance of the amino acid at position 591 of PB2 for efficient replication of the pandemic H1N1 viruses in humans has recently been reported (Yamada et al., 2012), and it was recorded that presence of basic amino acid at position 591 compensates for the lack of PB2-627K and substantially increased the lethality of an avian H5N1 virus in mice(Yamada et al., 2012). Presence of basic amino acid at position 591 not detected in strains under study.

The Identity Percent for the Amino Acid sequence of whole PB2 gene of the 5A/chicken/Egypt/ABD5/2015 isolates in comparison with A/chicken/Egypt/06553/2006 (H5N1) ranging between98% to 100%. And with the virus strain of A/Goose/Guangdong/1/96(H5N1) ranging between 98% and100percent.

In this study make partial analysis of pb1 and presence in one samples only mutation T672I.the partial analysis of pb1 idicate this mutation need further investigation to detect its impact

The Identity Percent for the Amino Acid sequence of whole PB1 gene of the 5 isolates in comparison with A/ chicken/ Egypt/ 06553/ 2006 (H5N1) ranging between 99% to 100 % and with the virus strain of A/ Goose/ Guangdong/ 1/ 96(H5N1) ranging between 98% and 100%.

In addition to the three polymerase proteins (PB-1, PB-2, and PA), the PB1 gene also codes for a fourth protein called the PB1-F2 (De Marco et al., 2003).The PB1-F2 protein is encoded by an alternative open reading frame of the PB1 RNA segment, PB1-F2 likely contributes to viral pathogenicity and might have an important role in determining the severity of pandemic influenza (García-Sastre A ., 2006).Theprotein is 87 amino acids long and the region coding for this protein is under intense selective pressure, although not found in all influenza A strains, it could certainly be considered a potential pathogenicity factor (Salomon et al., 2006), It was found that amino acid change (N66S) was found in the PB1-F2 sequence correlated with pathogenicity. This same amino acid change (N66S) was found in Hong Kong 1997 H5N1 outbreak and 1918 pandemic virus(Michael Lee, et al 2002).And this mutation is recorded in only one strain under study (A/Chicken/Egypt/115AD/2011(H5N1)) which is follow subclade2.2.1.1and it also present only in one Egyptian strain in 2007 (A/duck/Egypt/D3Lu220/2007(H5N1) ) , This protein has been shown to specifically target and destroy alveolar macrophages as it has an apoptotic induction effect on macrophages, thus reducing their ability to contribute to an immune response. It causes an inhibition of viral clearance, thus increasing cytotoxicity and increase pathogencity.

Also in PA V100A represent one of typical A.A signature of human influenza viruses observed in African strains was found in (A/CH/Egypt/1188-2011) strain under study. It was reported that mutations in the Amino acids T157A and 162, result in the loss of the potential phosphorylation site, weaken the proteolysis Function of PA. (Peralesetal 2000), that not recorded in our Egyptian strains. The polymerase PA sub unit considered a virulence factor for H5N1 AI virus in ducks. As it was reported that S224P in the PA protein dramatically increase the replication of H5N1 in ducks .This mutation not present in our Egyptian strains isolated from Duck, also N383D in PA plays an important role in the activity of the polymerase and in the accumulation of the polymerase PA and PB1 subunits in the nucleus of virus-infected cells, so increased the replication OF H5N1 virus in ducks. 383D present in all Egyptian strains (JiashengSong, et al 2011).

K356R detected in A/Ostrich/Egypt/11139F-2011 that considered one of typical AA signature of human influenza viruses observed in African strains (Guang-Wu Chen et al, 2006),also S409N recorded only in A/duck/Egypt/0871/2008

It was found that PA gene of all strains of subclade 2.2.1 (Egypt/2.2.1) containN341S, S388R, A448E and K615R. K615R represent one of important potential markers of host adaptation(Gabriel et al 2005).

In this study all the previous mutations but contain (S63G, I66T, V68I, V99I, N89S , I206V,K209R,R217C,N232K,K274K,S292A,G326N,T342A,M347L,D356E,K366R,R372K,R393S,K396R,E404K,T406P,E353A,I509L,K541R,E549R,A558E,V559I,L690P,L591F,R620K,R631K,S668P,A674V,L712F,A717Tand N721R) .

The Identity Percent for the Amino Acid sequence of whole PA gene of the 5 isolates in comparison with A/goose/Guangdong/1/1996 )A/bar-headed/Qinghai/3/2005) A/chicken/Israel/625/2006\ (H5N1) ranging between 96% to 100%.

NS1 protein is a multifunctional protein, the main function of NS1 protein is antagoniza the innate immunity of the infected host against avian influenza virus, by inhibition of IFN β synthesis, NS1 protein of Egyptian strains can be classified into 3 groups could be named A, B & C , each group has characteristic markers differ in between the groups as group (A) contain 44R , 48N and ESKV, group (B) contain 44K ,48N and ESKV while group (C)contain 44K,48S and ESEV.(Adel .A ,etal 2012).

In our study there are some mutations as:

(K49R,S53N,K60E,S181N,K76E,A81E,T165A,V203I,S221P,A230R,S234E).

the sample NO,2 contain another new mutations D263E,A61T,G229R .and sample NO 5A/ chicken/ Egypt/ ABD5/ 20 contain two mutation as I190VandF175S.

And it was found that Isolates under study are closely related to group C.

The amino acid S42 of NS1 is critical for the H5N1 influenza virus to antagonize host cell interfere and for the NS1 protein to prevent the double-stranded RNA mediated activation of the NF-β pathway and the IRF-3 pathway. All Egyptian isolates under study have amino acid serin (S) at the position 42. increased the replication and virulence in mammalian mice (Donelan et al., 2003)

Glutamic acid (E 92) of NS1of lethal H5N1 virus (HK/97) was resistant to the antiviral effects of IFN and TNF- α (Seo et al.,2002),The NS1 protein of this highly pathogenic virus has been shown to posses a unique 5 aa deletion at position 80 to 84 (15 nucleotides at position 238-252 nuclutides) resulting in a protein that is 5 a.a shorter than the normal 230 for avian influenza NS1s. An analysis of the public sequence databases reveals that this deletion emerged in H5N1 isolates in 2000 and has persisted ever since outlasting H5N1 viruses with a 230 aa NS1 protein..

the amino acid residue Ala149 (A) correlates with the ability of these viruses to antagonize interferon induction. the NS1 gene of GS/GD/1/96 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(Li, Z. et al, 2006)

but a A/goose/Guangdong/2/96 (GS/GD/2/96) virus encoding a Val (V) 149 substitution is not capable of the same effect.

In our study , all Egyptian isolates have Ala (A) amino acid at 149 in NS1 protein that give indication that the Egyptian strain can has high virulence in the infected different species recommendation for further studies on Egyptian H5N1 viruses isolates to learn more about host range and virulence determination .

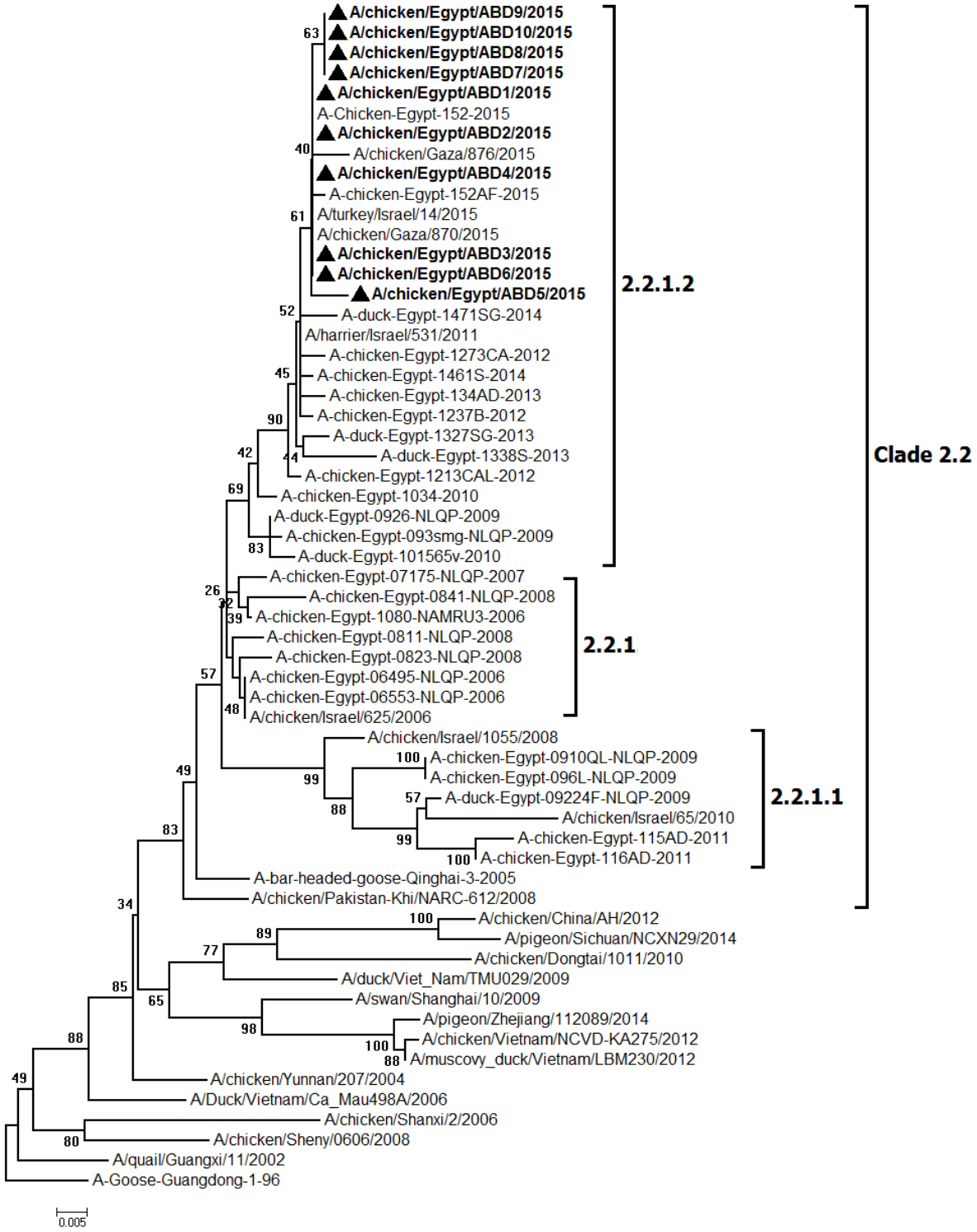
The Identity Percent for the Amino Acid sequence of NS gene of the 5 isolates in comparison with A/chicken/Egypt/06553/2006 and A/bar-headed/Qinghai/3/2005 ranging between 95 % to 100 % and with the virus strain of A/Goose/Guangdong/1/96(H5N1) ranging between 67.8 % and 69.3 %.

**CONCLUSION**

In our study we have investigated and characterized an HPAIV H5N1 and LPAIV H9N2 strain isolated from a retrospective analysis of a chicken backyard in Egypt from 2015 and compared the virus with selected reference viruses from Gen Bank. It is clear that HPAIV H5N1 isolates is closely related to classical strains belong to clade 2.2.2.1and there is no different changes with selected reference viruses from Gen Bank.

all Egyptian isolates have Ala (A) amino acid at 149 in NS1 protein that give indication that the Egyptian strain can has high virulence in the infected different species recommendation for further studies on Egyptian H5N1 viruses isolates to learn more about host range and virulence determination .

ISSN: 0975-8585




**FI(FIG 1)G. Phylogenetic tree of HA gene for selected Egyptian viruses.**

ISSN: 0975-8585

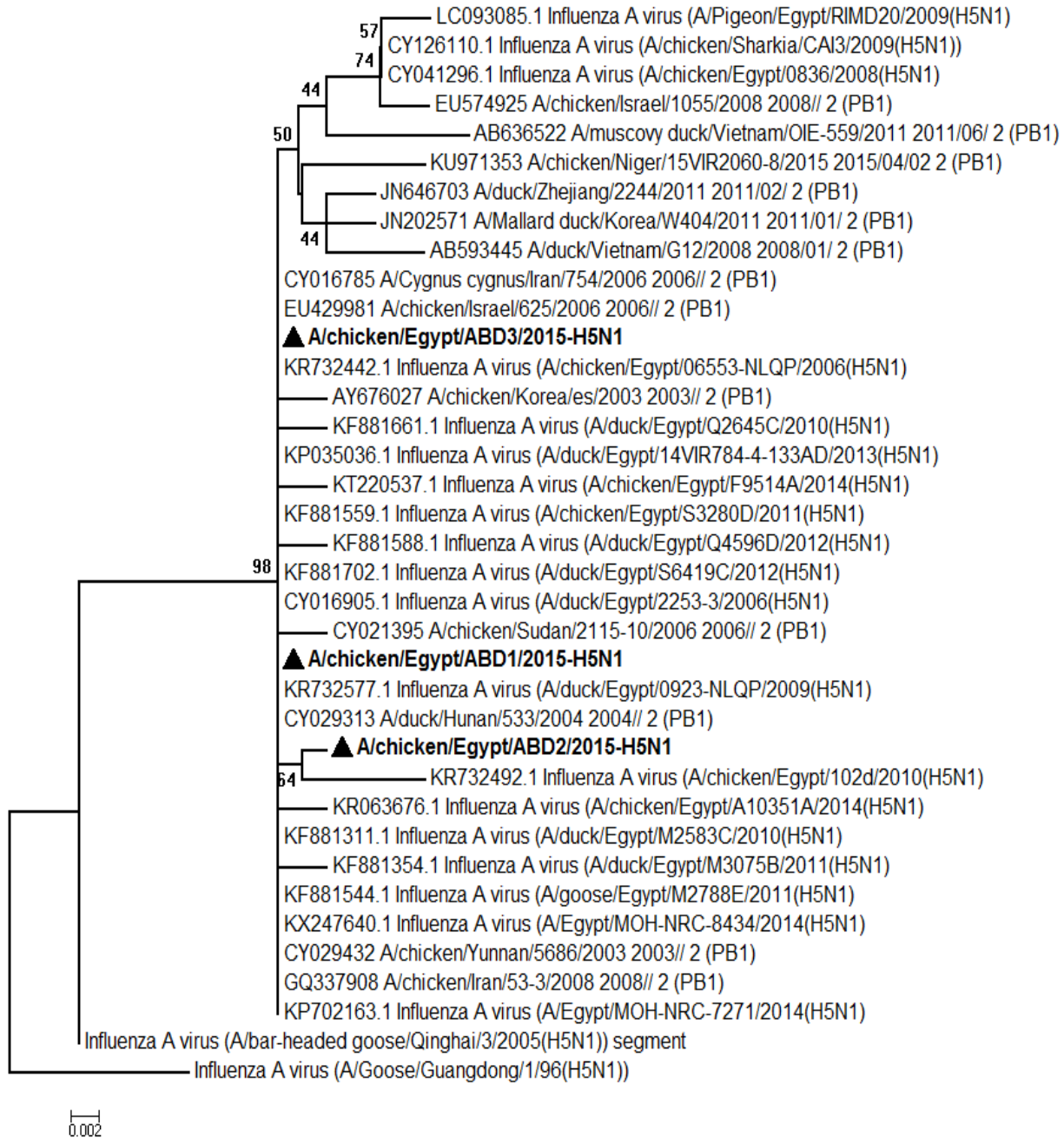



**FIG 2: Phylogenetic tree of PB1 gene for 5 selected Egyptian.**

ISSN: 0975-8585

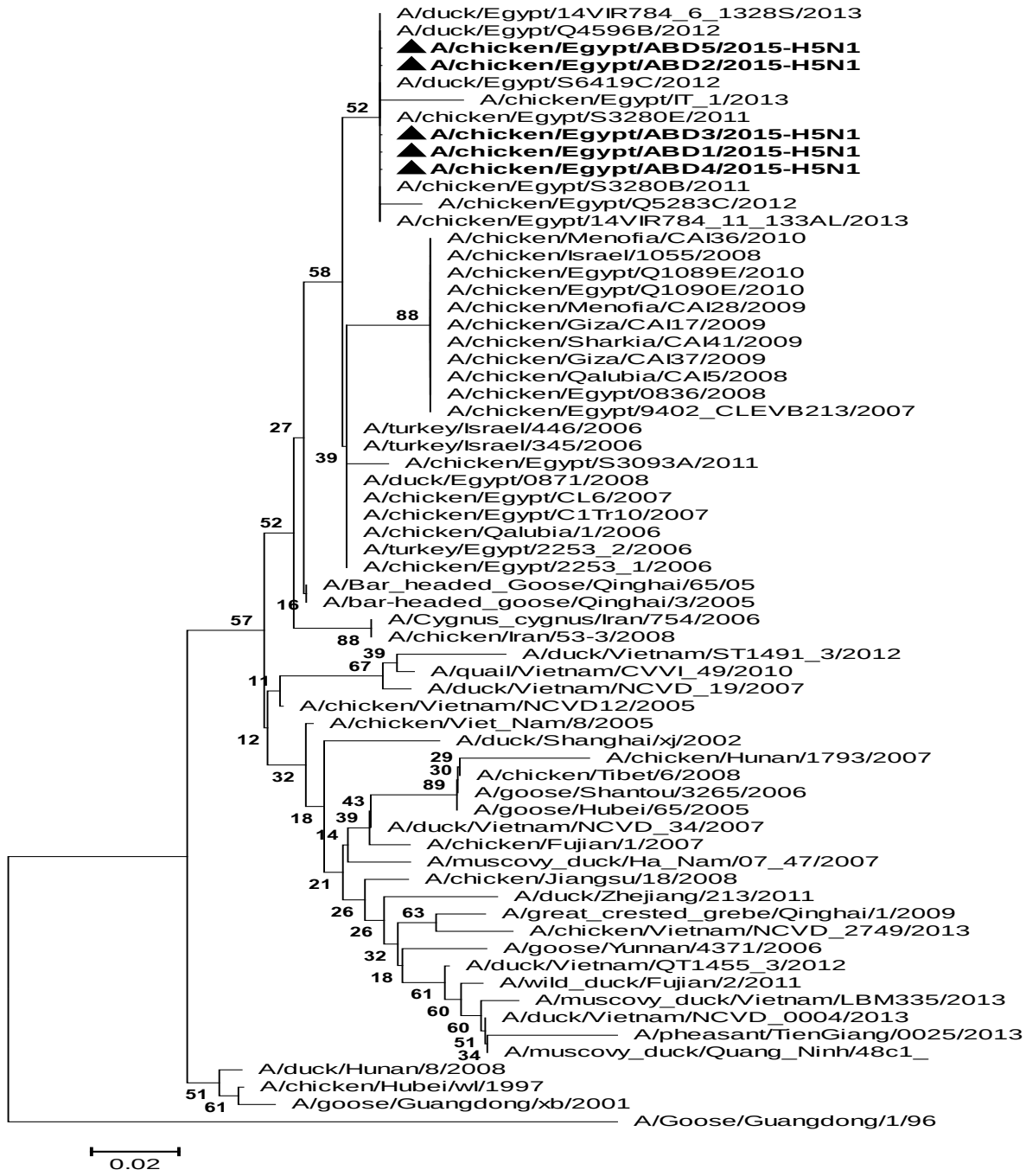



**FIG3: From the results of amino acid alignment and analysis of NS1 we found that all the Egyptian strains have deletion in 5 amino acids at position 80-84 in comparison to the A/Goose/Guangdong/1/96 strain**

ISSN: 0975-8585

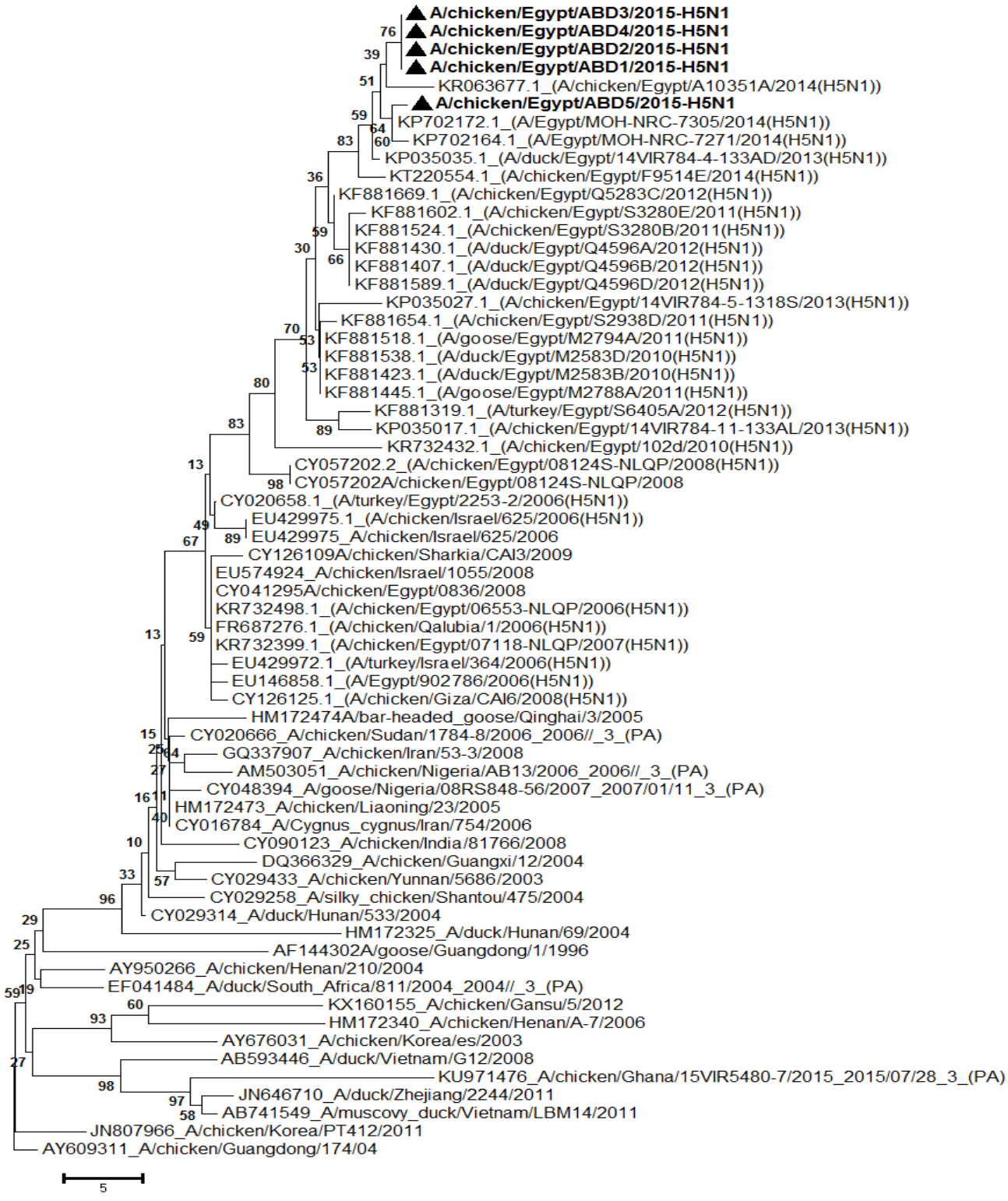



**FIG 4: Phylogenetic tree for the amino acid sequence of whole PA protein Fig (37) of the Egyptian isolates of the study and other Egyptian isolates from the gene bank in comparison with /Goose/Guangdong/1/96Isolate as a reference strain .Showing that here were two major groups (classic2.2.1andvariant2.2.1.1) circulated in this period.**

ISSN: 0975-8585

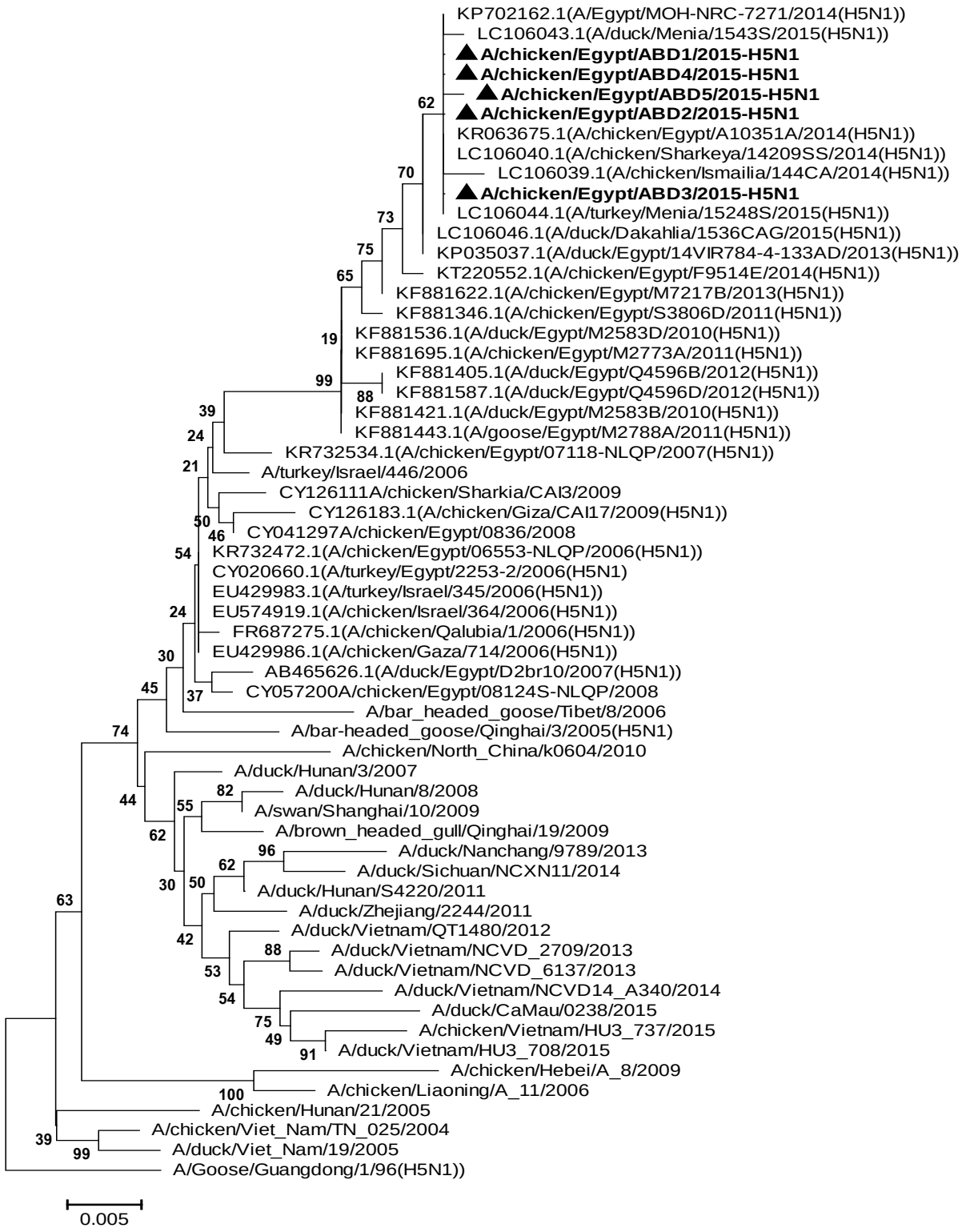



**FIG 5: Phylogenetic tree for the amino acid sequence of whole Pb2 gene of the Egyptian isolates under study in comparison with A/Goose/Guangdong/1/96Isolate as reference strain and other Egyptian isolates from the gene bank indicate a clustering of Egyptian viruses into two major groups (Egypt classic 2.2.1 and Egypt variant 2.2.1.1).**

ISSN: 0975-8585

**Supplementary table 2: the primers sets used in the amplification and sequence of the PB2, PB1, PA, HA and NS genes:**

| Primer ID | Nucleotide Sequence | Lengt | Reference |
|---|---|---|---|
| **PB2 Primers** | | | |
| **PB2-1FV2** | 5'GGAAAGCAGGTCAAATATATTCAATA 3' | 26 | **FLI** |
| **PB2-F-503** | 5'ATGGAGGTCGTTTTCCCAAATGAGG 3' | 25 | **NLQP** |
| **PB2-F910** | 5'TGAGGAACAAGCTGTGGATAT3' | 21 | **SEPRL** |
| **PB2-F1361** | 5'ATTGATAATGTCATGGGGATG 3' | 21 | **NLQP** |
| **PB2-1421R** | 5' GGT AAT AAT CCG ATC ATC CCC AT 3' | 23 | **NLQP** |
| **PB2-F-177** | 5'TAG TAC CAA AGG CTG CCA GA 3' | 20 | **NLQP** |
| **PB2-1RV2** | 5'GGTCGTTTTTAAACAATTCGACAC 3' | 24 | **FLI** |
| **PB2-R397** | 5'ACTTGGTTTCGGAAATGAAC3' | 20 | **NLQP** |
| **PB2-R828** | 5'TCTCCAGCAGTGATGCCAGTG 3' | 21 | **NLQP** |
| **PB2-R-231** | 5'CTA TTC GAC ACT AAT TGA TGG C 3' | 22 | **NLQP** |
| **PB1 Primers** | | | |
| **PB1-F-143** | 5'GCA AGC TAG TCG GAA TCA A 3' | 19 | **NLQP** |
| **PB1-R432** | 5'AATGTCCAGTCATAGGT CTG 3' | 20 | **NLQP** |
| **Pb1 R-867** | 5'CAC GAC GTT TGC CAA TTT AGC C 3' | 22 | **NLQP** |
| **PB1-1230R** | 5'ATGTTGAACATGCCCATCATCAT3' | 23 | **NLQP** |
| **PB1-R-232** | 5'TTC ATG AAG GAC AAG CTA A 3' | | **NLQP** |
| **PA Primers** | | | |
| **PA -3FV2** | 5'AGC GAA AGC AGG TAC TGA TTC AAA A 3' | 25 | **FLI** |
| **PA-F-450** | 5'CAC AGG GGA GGA AAT GGC AAC C3' | 22 | **NLQP** |
| **PA+734** | 5'AACCGAACGGCTGCATTGA 3' | 19 | **NLQP** |
| **PA-1131F** | 5'ACT CGG TGA GAA CAT GGC ACC3' | 21 | **NLQP** |
| **PA-F1825** | 5'ACATGACCAAAGAATTCTTTGA3' | 22 | **NLQP** |
| **PA-R-510** | 5'GCC CTG CTC TCT TCA TCA AGG G3' | 22 | **NLQP** |
| **PA-R-960** | 5'ATGTTGGGCTCCTTCCACCCGAAAAATG3 | 28 | **NLQP** |
| **PA -1312R** | 5'CTC CTA TTT CAT CAA GTT CTA TCC 3' | 24 | **NLQP** |
| **PA-R-1621** | 5'CTT TTC CCA CTT GTG TGG CTC C3' | 22 | **NLQP** |
| **PA-R-2193** | 5'ATA GTA GCA TTG CCA CAA CTA 3' | 21 | **NLQP** |
| **HA Primers** | | | |
| **Bm-HA-1** | TAT TCG TCT CAG GGA GCA AAA GCA GGG ( | 28 | **Hoffman, et al., 200** |
| **HGGT+** | CTC TTC GAG CAA AAG CAG GGG T | 22 | **SEPRL** |
| **H5F3-395** | AAC ACC TAT TGA GCA GAA TAA A | 22 | **NLQP** |
| **KH1-800** | CCT CCA GAR TAT GCM TAY AAA ATT GTC | 27 | **VLA** |
| **NS Primers** | | | |
| **NS-8FV2** | **5'**AAA AGC AGG GTG ACA AAG ACA TAA **3'** | 24 | **FLI** |
| **NS-8RV2** | **5'**AGT AGA AAC AAG GGT GTT TTT TAT CA **3** | 26 | **FLI** |
| **NS-R598** | **5'**GAG TTA TCA TTC CAT TCA AGT CC **3'** | 23 | **SEPRL** |
| **NS-F327** | **5'**GACTGG TTC ATG CTC ATG CC **3'** | 20 | **SEPRL** |

| **HA Primers** | | | |
|---|---|---|---|
| Bm-HA-1 | TAT TCG TCT CAG GGA GCA AAA GCA GGG G | 28 | Hoffman, et al 2001 |
| HGGT+ | CTC TTC GAG CAA AAG CAG GGG T | 22 | SEPRL |

ISSN: 0975-8585

| | | | |
|---|---|---|---|
| H5F3-395 | AAC ACC TAT TGA GCA GAA TAA A | 22 | NLQP |
| KH1-800 | CCT CCA GAR TAT GCM TAY AAA ATT GTC | 27 | VLA |
| H5 F4-817 | AGT AAT GGA AAT TTC ATT GCT CCA GAA | 27 | NLQP |
| H5-951 F | AAT GYC CCA AAT AYG TGA AA | 19 | NLQP |
| H5F5-1088 | TTG GAG CTA TAG CAG GTT TTA TAG AGG | 27 | NLQP |
| H5F6-1352 | TCT GGA CTT ATA ATG CTG AAC T | 22 | NLQP |
| Bm-NS-890R | ATA TCG TCT CGT ATT AGT AGG AAA CAA GGG TGT TTT | 36 | Hoffman et al., 200 |
| H5R3-1352 | AAG TTC AGC ATT ATA AGT CCA GA | 23 | NLQP |
| H5R4 -1085 | CCT CTA TAA AAC CTG CTA TAG CTC CAA ATA | 30 | NLQP |
| H5R5-817 | TTC TGG AGC AAT GAA ATT TCC ATT ACT | 27 | NLQP |
| H5-R16-460 | ATG CTG AGC TCA CTC CTG ATG | 21 | NLQP |

**Supplementary table 3: Accession no. of the sequenced genes HA, PB1, PB2, PA and NS of the isolates in this study:**

| Isolate ID | HA | PB1 | PB2 | PA | NS |
|---|---|---|---|---|---|
| A/Chicken/Egypt/ABD1/2015 | | | | | |
| A/Chicken/Egypt/ABD2/2015 | | | | | |
| A/Chicken/Egypt/ABD3/2015 | | | | | |
| A/Chicken/Egypt/ABD4/2015 | | | | | |
| A/Chicken/Egypt/ABD5/2015 | | | | | |
| A/Chicken/Egypt/ABD6/2015 | | | | | |
| A/Chicken/Egypt/ABD7/2015 | | | | | |
| A/Chicken/Egypt/ABD8/2015 | | | | | |
| A/Chicken/Egypt/ABD9/2015 | | | | | |
| A/Chicken/Egypt/ABD10/201 | | | | | |

## REFERENCES


[1] Zhou, N. N.;Shortridge, K.F.;Claas, E.C.; Krauss, S.L. and Webster, R.G. (1999):

[2] Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong.J.Virol., (73): 3366–3374 Spackman et al., 2002

[3] Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin

[4] subtypes. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992):Evolution and ecology of influenza A viruses MicrobiolRev 56:152–179

[5] Swayne, D. E. and Halvorson, D. A., (2003): Avian influenza in Diseases of Poultry, Eleventh Edition; Saif Y.M., Barnes H.J.; Gilson J.R., Fadly A.M., Mcdougald I.R., and Swayne D.E. eds. Iowa State University Press, Iowa, USA, 135-160.

[6] Suarez D. L., A E. Spackman,A and D. A. Senne(2003):Update on Molecular Epidemiology of H1, H5, and H7 Influenza Virus Infections in Poultry in North America .Avian diseases 47:888–897, 2003

[7] Swayne, D. E. and Halvorson, D. A., (2003): Avian influenza in Diseases of Poultry, Eleventh Edition; Saif

Y.M., Barnes H.J.; Gilson J.R., Fadly A.M., Mcdougald I.R., and Swayne D.E. eds. Iowa State University Press, Iowa, USA, 135-160.

[8] Swayne, D. E. and Halvorson, D. A. (2003)

[9] Avian influenza.in Diseases of Poultry, Eleventh Edition; Saif Y.M., Barnes H.J.; Gilson J.R., Fadly A.M., Mcdougald I.R., and Swayne D.E. eds. Iowa State University Press, Iowa, USA, 135-160.

[10] Aly, M.M.; Arafa, A.; Hassan, M.K.; Selim, A.A.and E.M. Abdelwhab(2007-a):Serological investigation on broiler immune response to avian influenza H5 vaccines in Egypt. Proceeding of the 7th international conference of faculty of veterinary medicine, Cairo university .Aly, M.M.; Hassan, M.K. and A. Arafa (2006-a): Emergence of highly pathogenic H5N1 avian influenza virus in poultry in Egypt. First record of 2006 outbreaks. Journal of the Egyptian veterinary medical association, 66(2): 263-276.

[11] Aly, M.M.; Kanawaty, Z.; Arafa, A.; Kilany, W.H. and E.M.Abdelwhab(2007-b):-One-year surveillance on avian influenza H5N1 in backyard poultry in Egypt. 4th international symposium of turkey production.Berlin 21-23 June .Aly, M. M.; Arafa, A.; Kanawaty, Z.; Abdel-Whab. E. M. and Hassan, M.K. (2008-b):Avian Influenza H7 surveillance on backyard birds around El-Abassa Lake in Egypt, 2007. In the Second International Conference of Virology, Emerging and Exotic Viral Infection Challenging Threats of Human, Animal and Plant Health 5-6 April, 2008. Organized by Egyptian Society of Virology, Cairo, Egypt. Aly, M.M.; Arafa, A. and Hassan, M.K. (2008-a).Epidemiological Findings of Outbreaks of Disease Caused by Highly Pathogenic H5N1 Avian Influenza Virus in Poultry in Egypt during 2006. Avian Dis., (52): 269–277

[12] AbdelMoneim, AhmedS, Salama A. S. Shany, Sasan R. Fereidouni, Bahaa T. M. Eid,Magdy F. El-Kady, Elke Starick, Timm Harder and Günther M. Keil.(2009):Sequence diversity of the haemagglutinin open reading frame of recent highly pathogenic avian influenza H5N1 isolates from Egypt, Archivef of virology (2009) Volume: 154, Issue: 9, Pages: 1559-1562

[13] Abdel-Moneim AS, Afifi MA, El-Kady MF (2012):Isolation andmutation trend analysis of influenza A virus subtype H9N2 inEgypt. Virol J 9:173

[14] Abdelwhab E.M. and Hafez H.M. (2011): An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: epidemiology and control challenges. Epidemiol. Infect. (2011), 139, 647–657.

[15] Shabat, M. Ben, Meir, R., Haddas, R., Lapin, E., Shkoda, I., Raibstein, I., Perk, S., Davidson, I., 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J. Virol. Methods 168, 72–77.

[16] Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, in: Nucleic Acids Symposium Series. [London]: Information Retrieval Ltd., c1979-c2000., pp. 95–98.

[17] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

[18] Gupta, R., Brunak, S., 2001. Prediction of glycosylation across the human proteome and the correlation to protein function, in: Biocomputing 2002. World Scientific, pp. 310–322.

[19] Yang, Z., 1996. Maximum-likelihood models for combined analyses of multiple sequence data. J. Mol. Evol. 42, 587–596.

[20] Rambaut, A., 2009. FigTree, version 1.3. 1. Comput. Progr. Distrib. by author, website http//treebioedacuk/software/figtree/[accessed January 4, 2011].

[21] Rambaut, A., Drummond, A.J., 2009. Tracer version 1.2 [computer program].Arafa, A. ; Selim, A. A. ; Hassan, M. K. and Aly, M.M. (2008): Epidemiological surveillance on avian influenza virus H5N1 infection in poultry in 2007.Proceeding of the Ninth Scientific Conference of Egyptian Veterinary poultry association, 10-12 March 2008, Cairo, Egypt.

[22] Arafa, A.; Suarez, D.L.; Hassan, M. K. and Aly, M.M. (2010-b): Phylogenetic analysis of HA and NA genes of HPAI-H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogenicity with multiple distinct sublineages.Avian Diseases 54(s1):345-349. 2010

[23] Hoffmann, E.;Stech ,J.; Guan ,Y.; Webster, R.G. and Perez, D.R.(2001): Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. ,146(12):2275–2289

[24] Neumann, G., Kawaoka, Y., 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg. Infect. Dis. 12, 881.

[25] Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, et al. (2005) The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. ProcNatlAcadSci U S A 102: 18590–18595

[26] Gabriel G, Herwig A, Klenk HD (2008) Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoSPathog 4: e11.

[27] Salomon R., Franks J., Govorkova E. A., Ilyushina N. A., Yen H. L., Hulse-Post D. J., Humberd J., Trichet M., Rehg J. E., Webby R. J., et al. (2006).. The polymerase complex genes contribute to the high virulence of

the human H5N1 influenza virus isolate /Vietnam/1203/04. J. Exp. Med. 203:689–697

[28] Poole E. L .Medcalf L., Elton D., Digard P.2007Evidence that the C-terminal PB2-binding region of the influenza A virus PB1 protein is a discrete alpha-helical domain. FEBS Lett 581, 5300–5306.

[29] Hatta M, Gao P, Halfmann P, Kawaoka Y (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293: 1840–1842. Find this article online

[30] Bussey, K. A., T. L. Bousse, E. A. Desmet, B. Kim, and T. Takimoto. 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host Cells. J. Virol. 84:4395–4406.

[31] Shaw M.L., Stone K.L., Colangelo C.M., Gulcicek E.E., Palese P. (2008)

[32] Cellular Proteins in Influenza Virus Particles. PLoSPathog 4(6): e1000085. doi:10.1371/journal.ppat.1000085. Editor: Klaus Früh, Oregon Health & Science University, United States of America. Received: December 6, 2007; Accepted: May 7, 2008; Published: June 6, 2008

[33] Adel, A., Arafa, A., Hussein, H.A., El-Sanousi, A.A., 2017. Molecular and antigenic traits on hemagglutinin gene of avian influenza H9N2 viruses: Evidence of a new escape mutant in Egypt adapted in quails. Res. Vet. Sci. 112, 132–140. doi:10.1016/j.rvsc.2017.02.003

[34] Gao, H., Xu, G., Sun, Y., Qi, L., Wang, J., Kong, W., Sun, H., Pu, J., Chang, K.-C., Liu, J., 2015. PA-X is a virulence factor in avian H9N2 influenza virus. J. Gen. Virol. 96, 2587–2594.

[35] Wang, J., Sun, Y., Xu, Q., Tan, Y., Pu, J., Yang, H., Brown, E.G., Liu, J., 2012. Mouse-adapted H9N2 influenza A virus PB2 protein M147L and E627K mutations are critical for high virulence. PLoS One 7, e40752.

[36] Li, Z.; Chen, H.; Jiao, P.; Deng, G.; Tian, G.; Li, Y.; Hoffmann, E.; Webster, R.G.; Matsuoka, Y. and K. Yu (2005):Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J. Virol., 79:12058–12064.

[37] Li, Z.; Jiang, Y.; Jiao, P.; Wang, A.; Zhao, F.; Tian, G.; Wang, X.; Yu, K.; Bu, Z. and H. Chen (2006): The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J. Virol., 80:1115–1123.

[38] Duvvuri VRSK, Duvvuri B, Cuff WR, Wu GE, Wu J(2009):Role of positive selection pressure on the evolution of H5N1 hemagglutinin. Genomics, Proteomics and Bioinformatics 2009, 7:47-56.

[39] Peiris JS, de J, Guan Y (2007) :-Avian influenza virus (H5N1): a threat to humanhealth. ClinMicrobiol Rev 20: 243–267.

[40] Lycett S. J., Ward M. J., Lewis F. I., Poon A. F. Y., Kosakovsky Pond S. L., Leigh Brown A. J. (2009) Detection of mammalian virulence determinants in highly pathogenic avian influenza H5N1 viruses: multivariate analysis of published data J. Virol.83 (19):9901-9910 Doi:10.1128/JVI.00608-09 - Abstract - Full Text.

[41] Yamada S, Masato Hatta, Bart L. Staker, Shinji Watanabe, Masaki Imai, Kyoko Shinya, Yuko Sakai-Tagawa, Mutsumi Ito, Makoto Ozawa, Tokiko Watanabe, Saori Sakabe1, Chengjun Li, Jin Hyun Kim, Peter J. Myler, Isabelle Phan, Amy Raymond , Eric Smith, Robin Stacy, Chairul A. Nidom, Simon M. Lank, Roger W. Wiseman, Benjamin N. Bimber, David H. O'Connor, Gabriele Neumann , Lance J. Stewart, Yoshihiro Kawaoka 2012.Biological and Structural Characterization of a Host-Adapting Amino Acid in Influenza Virus. 2012 Jul 13;337(6091):199-204. doi: 10.1126/science.1222213. Epub 2012 Jun 28

[42] García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D.E., Durbin, J.E., Palese, P., Muster, T., 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 252, 324–330.

[43] Michael Lee M. T, Konrad Bishop, Liz Medcalf, Debra Elton, Paul Digard, and Laurence Tiley (January 15, 2002)."Definition of the minimal viral components required for the initiation of unprimed RNA synthesis by influenza virus RNA polymerase". Nucleic Acids Research 30 (2): 429–438.

[44] Guan, Y. and Chen, H. (2005): Resistance to anti-influenza agents. Lancet 366,1139–1140

[45] Guang-Wu Chen,Shih-Cheng Chang, Chee-KengMok, Yu-Luan Lo, Yu-Nong Kung, Ji-Hung Huang, Yun-Han Shih, Ji-Yi Wang, hiaynChiang, Chi-Jene Chen, and Shin-Ru Shih (2006 )Genomic Signatures of Human versus Avian Influenza A Viruses Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P 2013An overlapping protein-coding region in influenza A virus segment 3 modulates the host response , Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, UK Perdue, M.L. 1992.Naturally occurring NS genevariants in an avian influenza virus isolate. VirusResearch 23:223–240.

[46] Stallknecht, D.E., M.T. Kearney, S.M. Shane,and P.J. Zwank. 1990. Effects of pH, temperature,and salinity on persistence of avian influenzaviruses in water. Avian Diseases 34:412–418.

[47] Perkins, L.E.L., and D.E. Swayne.(2002). Susceptibilityof Laughing Gulls (Larusatricilla) toH5N1 and H5N3 highly pathogenic avian influenzaviruses. Avian Diseases 46:877–885

[48] Perkins, L.E.L., and D.E. Swayne. (2003). Variedpathogenicity of a Hong Kong-origin H5N1 avian influenza virus in four passerine species and budgerigars.Veterinary Pathology 40:14–24.

[49] Rosenberger, J.K., W.C. Krauss, and R.D.Slemons (1974). Isolation of Newcastle-diseaseand type-A

influenza viruses from migratorywaterfowl in Atlantic Flyway. Avian Diseases18:610–613.

[50] Wang, W.; Riedel, K.; Lynch, P.; Chien, C.Y.; Montelione, G.T. and R.M. Krug (1999): RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA, 5: 195–205.

[51] Seo, S.H., Hoffmann, E., Webster, R.G., 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8, 950.

[52] Dundon G. and CapuaI. ( 2009) , A Closer Look at the NS1 of Influenza Virus. (2009) OIE/FAO and National Reference Laboratory for Avian influenza and Newcastle Disease, IstitutoZooprofilatticoSperimentaledelleVenezie, Viale dell' Università, 10, Legnaro (PD), 35020, Italy; E-Mail: icapua@izsvenezie.

[53] Marsh, G. A., Rabadan, R., Levine, A. J. &Palese, P. (2008).Highly conserved regions of influenza A virus polymerase gene segments are critical for efficient viral RNA packaging. J Virol 82, 2295–2304.

[54] ElHefnawi M , Osama AlAidi, Nafisa Mohamed, Mona Kamar, Iman El-Azab, SuherZada, Rania Siam 2011. Identification of novel conserved functional motifs across most Influenza A viral strains ,Virology Journal 2011, 8:44.

[55] Balannik, V., Carnevale, V., Fiorin, G., Levine, B.G., Lamb R.A., Klein, M.L., DeGrado, W.F. and Pinto, L.H. (2010): Functional studies and modeling of pore-lining residue mutants of the influenza a virus M2 ion channel. Biochemistry 2010, 49:696-708.

[56] Fusaro A, Nelson MI, Joannis T, Bertolotti L, Monne I, et al. (2010) Evolutionary dynamics of multiple sublineages of H5N1 influenza viruses in Nigeria from 2006 to 2008. J Virol 84: 3239–3247. Find this article online.

[57] Fusaro A, Monne I, Salviato A, Valastro V, Schivo A, AmarinNM, Gonzalez C, Ismail MM, Al-Ankari AR, Al-BlowiMH,Khan OA, Maken Ali AS, Hedayati A, Garcia Garcia J, ZiayGM,Shoushtari A, Al Qahtani KN, Capua I, Holmes EC, Cattoli G(2011):Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol 85:8413–8421Solimanetal.,2014a