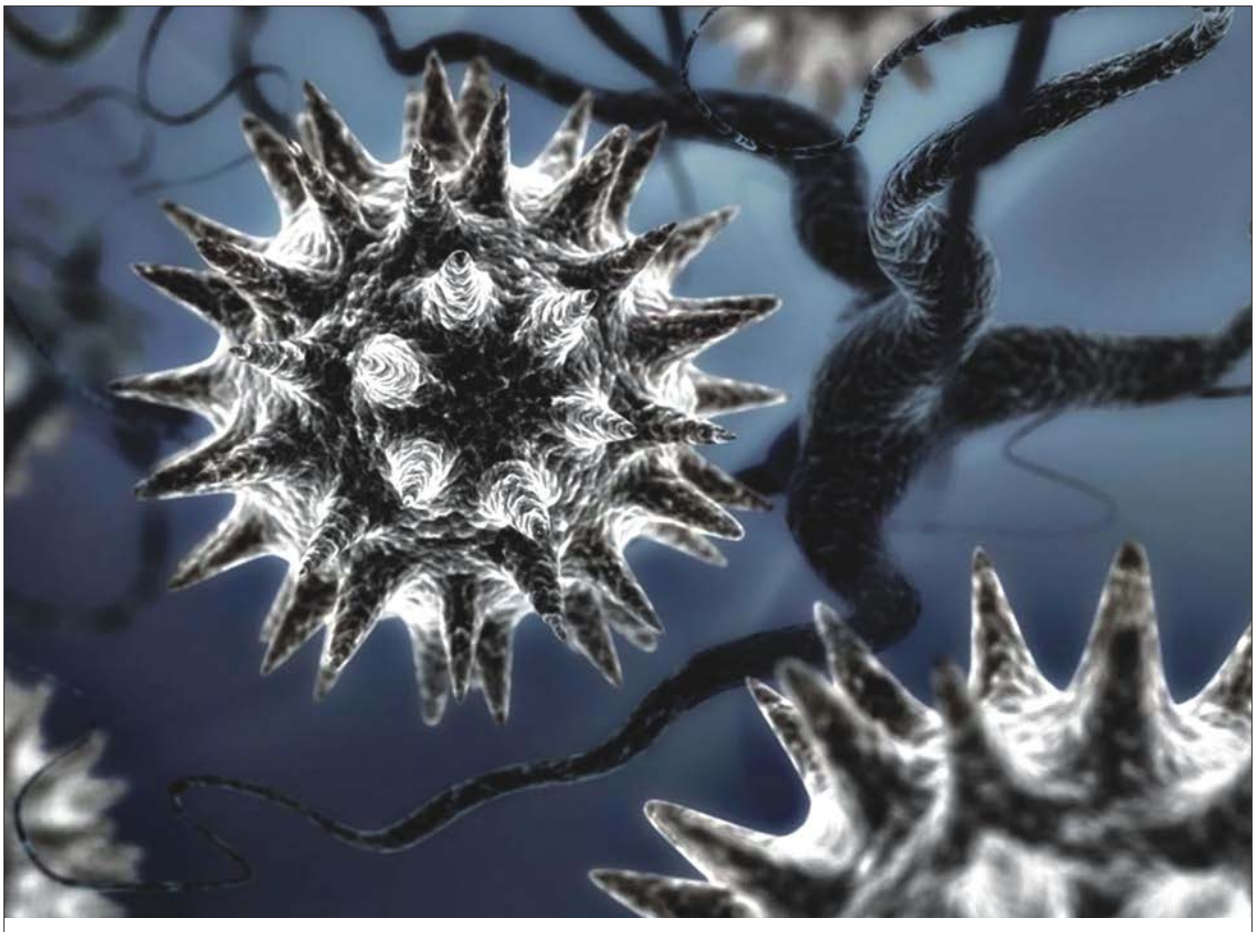

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Molecular Characterization of Highly Pathogenic *Avian influenza* H5N1 in Ostrich

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ABSTRACT

The highly pathogenic *Avian influenza* virus H5N1 (HPAIV-H5N1) represents an important poultry pathogen and a major *havoc* to the poultry industry. Furthermore, H5N1 infections in poultry constitute a threat to mammals including humans. Egypt has been affected by a HPAI H5N1 since 2006 and the virus still circulates in poultry population until now. Among the great number of species affected by the virus, ostrich have been occasionally infected worldwide. In the present study, we report a detailed molecular characterization of H5N1 virus isolated from ostrich farm during 2010 and study the virulence markers and other point mutations in HA, NA and other internal genes and proteins associated with traits such as specific-host receptor affinity and some antiviral resistance markers.

Key words: *Avian influenza*, HSNI, pathogen, poultry induster, ostrich, RNA, genome

INTRODUCTION

Avian influenza viruses (AIV; family Orthomyxovidae) are segmented single-stranded negative-sense RNA genome. The eight genomic segments encode the Hemagglutinin (HA), Neuraminidase (NA), Nonstructural (NS), Matrix (M), Nucleocapsid protein (NP), Polymerase basic 1 (PB1), Polymerase Basic 2 (PB2) and Polymerase Acidic (PA) Genes (Alexander, 2000; Webster *et al*., 1992) some of which contain molecular virulence markers and other specific point mutations in internal proteins associated with host receptor affinity and antiviral resistance.

Clinical signs, severity and lesions that have been reported in ostriches are variable and have been shown to be influenced by a number of factors. These factors include age, concurrent infection especially with *E. coli*, *Pseudomonas aeruginosa*, *Aspergillus fumigatus* and *Staphylococcus aureus*. Clinical signs include depression, respiratory distress, ocular discharges and green discoloration of urine. Other signs are ruffled feathers and complications arising from secondary infections. Younger birds are more severely affected than mature ones. Mortality rate is also variable, where in young ostriches it reaches up 60-80% and high mortality may be first indication of presence of disease in a flock (Huchzermeyer, 1998).

The pathogenicity of H5N1 influenza viruses in ostriches is considered to be multigenic and the primary virulence factor is the presence of multiple basic amino acids or the insertion of amino acids

at the HA cleavage site (Horimoto and Kawaoka, 1994; Senne *et al*., 1996). In addition to the HA cleavage site, a deletion of 20 amino acids in Stalk region of NA gene previously studied to increase virulence and a lysine residue at amino acid 627 in PB2 has been found in most influenza viruses pathogenic to humans and also influences virulence in mice (Hatta *et al*., 2001; Munster *et al*., 2007).

Sequence analysis of the viral genes and their respective amino acids is a valuable tool for detection of any antigenic variation among viruses circulating in the field and also provides information about the source of the initial of the virus has resulted in a progressive genetic evolution and an antigenic drift (Cattoli *et al*., 2011).

Initially the sequenced data of the Egyptian *Avian influenza* viruses revealed that all Egyptian strains were very closely related and belonging to clade 2.2 of the H5N1 virus of Eurasian origin. In present, according to WHO/OIE/FAO H5N1 evolution working group, highly Pathogenic *Avian influenza* viruses circulating in Egypt are belonging to clade 2.2.1 and clade 2.2.1.1. evolving strains from 2006 till 2011 (WHO *et al*., 2009).

In this study, we focused on molecular characterization of H5N1 virus isolated from ostrich farm during 2010 to study the virulence markers and other mutations in HA, NA and other internal genes and proteins.

MATERIALS AND METHODS

Cloacal and tracheal swabs were collected through NLQP (National Laboratory of Veterinary Quality Control on Poultry Production). Detection and identification of the subtype H5 *Influenza virus* was done by Real-Time RT-PCR, using Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA): Cat No. 204443 and specific primers and probe for the H5 subtype, Primer and Probe according to Spackman *et al*. (2002). Probe H5 5-Fam TCWACAGTGGCGTTCCCTAGCA-Tamra Q and H5 primers are H5 LH1 (24 bp) 5' ACG TAT GAC TAC CCG CAG TAT TCA 3' and H5 RH 1 (20 bp) 5' AGA CCA GCC ACC ATG ATT GC 3'. The reaction was carried out by use of the programme: Reverse transcription step 50°C 30 min for 1 cycle, Heat activation of polymerase step 95°C 15 min for 1 cycle, PCR denaturation 94°C 10 sec, Annealing 54°C 30 sec and Extension 72°C 10 sec. Steps are repeated for 40 cycles in Stratagen MX3005P Real-Time PCR machine (Stratagene, USA) then Propagation on SPF ECE was done at 9-11 days by Intra-allantoic method (OIE, 2008) The eggs were incubated at 37°C for 5 days with daily examination then collection of allantioc fluid from the eggs. The haemagglutination test was applied on the collected allantoic fluid (OIE, 2009), Viral RNA were extracted from the infective allantoic fluid using QIAmp viral RNA mini kit and were reverse transcribed with the Access Quick RT-PCR using UN1-12 primer, PCR amplification was performed by using specific primers for each gene of AI (HA, NA, PB2, PB1, PA, NP, NS and M) (primer sequences available on request) using Reddy master mix (Thermo two-step) using different annealing temperatures according to the melting TM of each primer. The sequences were generated using the Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA-USA). The products of the sequencing reactions were cleaned-up using Centrisep purification kit (Applied Biosystem, CA-USA) and sequenced in a 4 capillary ABI PRISM 3130 Genetic Analyzer (Applied Biosystem, CA-USA). Sequences from all eight gene segments were aligned and compared with A/H5N1 sequences of viruses from Egypt and with representative sequences of viruses from the Middle East available on GenBank. Phylogenetic analyses were carried out for all the eight gene segments using the neighbour joining (N-J) method with laser gene software (DNA Star).

Virus used in this study: A/Ostrich/Egypt/11139F/2011 (H5N1) collected, identified and sequenced in (NLQP).

RESULTS

Result of virus propagation: Allantoic fluids were harvested from eggs died during the period of incubation and from eggs surviving up to 120 h post inoculation (PI) then were tested for presence of haemagglutinating agent using the HA test, the HA titer is 8 Log 2.

Results of RT-real time PCR: Use a set of primers and Taqman probe mentioned in materials and methods for H5 gene detection using Quantitect real time RT-PCR kit, Positive control is a sample previously tested for H5N1 and show high positivity for H5. The result of real time PCR was shown in Fig. 1 and the (ct. value) is 16.

Ct. value: The point at which the fluorescence crosses the threshold.

Molecular characterization of surface glycoprotein genes: Based on the deduced amino acid sequence of the H5N1 strain isolated from ostrich flock it was found that this strain belong to clade 2.2.1 that is of Eurasian origin and contained multi basic amino acid motif PQGEKRRKKRGLF at their HA cleavage sites, which is a Characteristic of highly pathogenic *Avian influenza* virus (Peiris *et al*., 2007; Chen *et al*., 2006; Li *et al*., 2004), beside presence of glycosylation patterns similar to that of the highly pathogenic H5 viruses.

There is a deletion in amino acid No. 129 which considered a part from receptor binding site and also belongs to an antigenic site (epitope A) and not recorded in the ancestor A/GOOSE/Guangdong/1/96 strain. Ostrich strain also contain S132R substitution mutation that detected in receptor binding domain, or close proximity to it.

Compared with the NA of A/goose/Guangdong/1/96, ostrich isolate contain a 20 amino acid deletion in the stalk of NA Molecules, which is identical to the genotypes V and Z (Peiris *et al*., 2007; Chen *et al*., 2006; Li *et al*., 2004), The substitutions 274 (H to Y) and 294 (N to S) were reported to Confer resistance to oseltamivir in clinical influenza (H5N1) isolates (Le *et al*., 2005). No similar amino acid substitutions were observed at the Conserved residues in the NA of ostrich strain. These Results suggest that the isolated virus is sensitive to NA inhibitors.

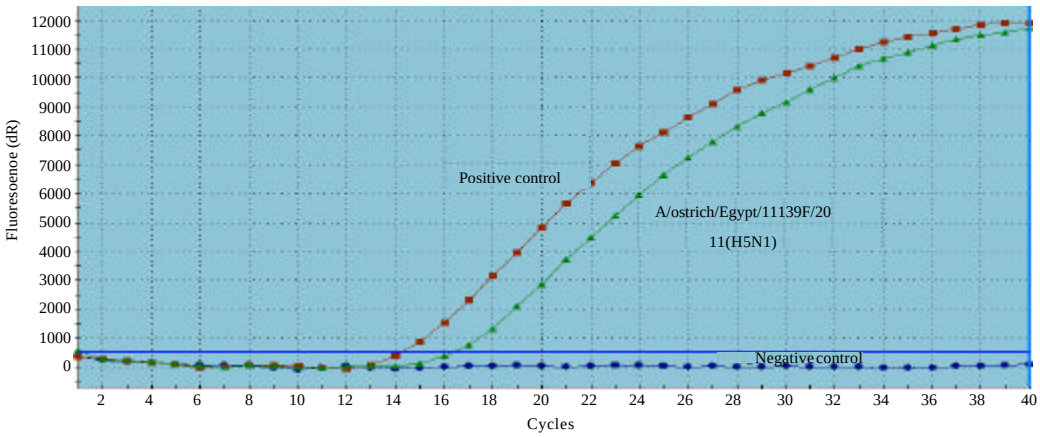

Fig. 1: Amplification curves of real time PCR

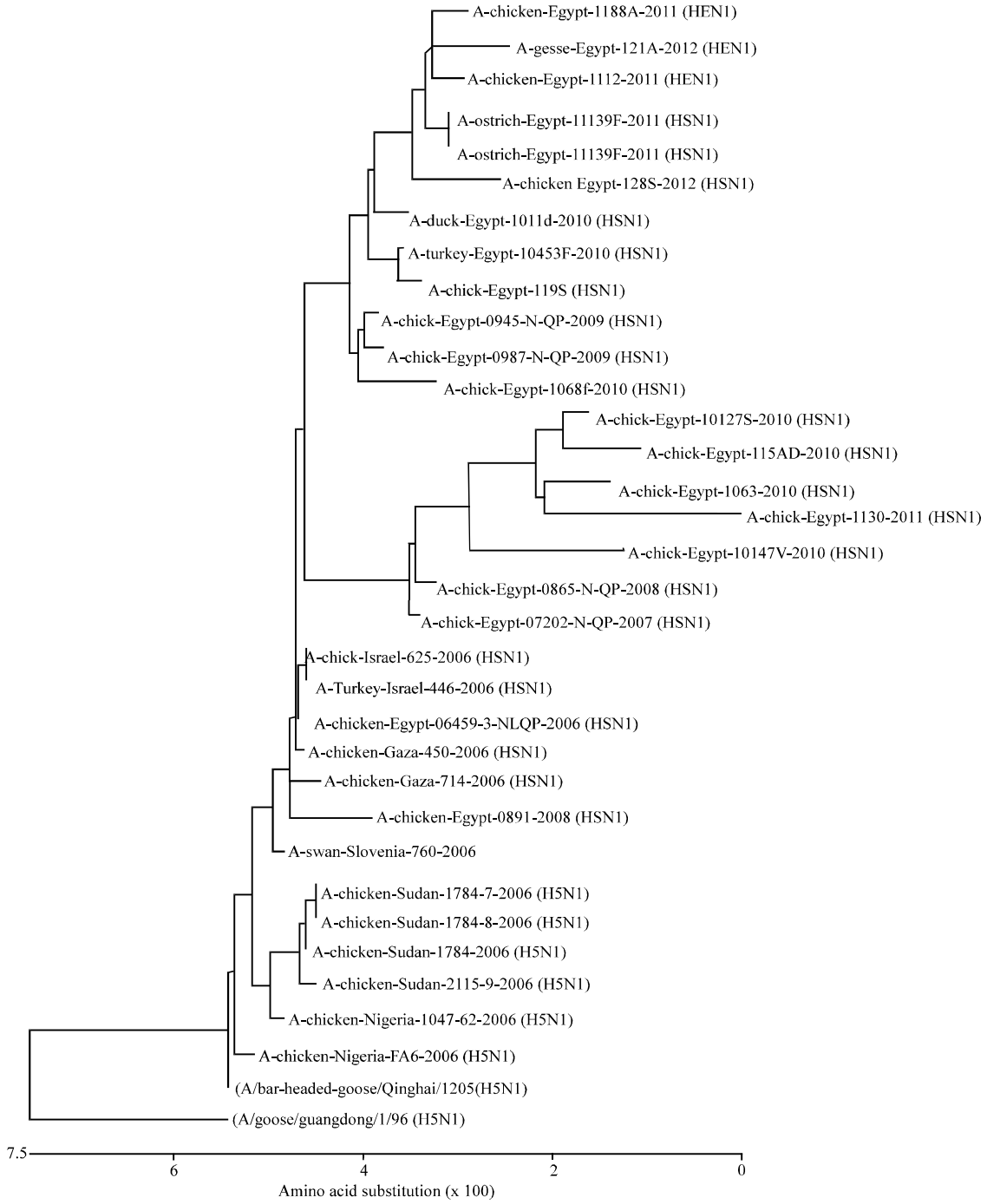

Fig. 2: Phylogenetic tree analysis of HA amino acid sequence of A/Ostrich/Egypt/11139F/2011

Phylogenetic tree for the HA amino acid sequence of A/Ostrich/Egypt/11139F/2011 in comparison to A/Goose/Guangdong/1/96 (Fig. 2).

Phlogenetic analysis of HA gene of A/Ostrich/Egypt/11139F/2011 showed that ostrich strain is closely related to H5N1 strain isolated in 2006. Furthermore, Israeli and Gazian viruses were closely related to viruses isolated in Egypt.

Table 1: Amino acid comparison of PB2, PB1, HA, NA and NS of A/ostrich/Egypt /11139F/2011 and A/goose-guangdong/1/96

	PB2	PB1		HA[1]				NA[2]		NS	
Virus	627	13	436	129	226	228	C.S	49-68	294	80-84	PDZ[3]
A/goose/guangdong/1/96	e	P	Y	+	Q	G	PQRERRRKKRGLF	+	N	+	ESEV
A/ostrich/Egypt/11139F/2011	K	P	Y	-	Q	G	PQGEKRRXKKRGLF	-	N	-	ESEV

[1]H3 numbering, [2]N2 numbering, [3]PDZ binding domain, +: No amino acid deletion, -: Amino acid deletion

Molecular characterization of polymerase and NP genes: Several amino acid mutations of the polymerase (PB2, PB1 and PA) and NP protein may have an important effect on the virulence and adaptation of H5N1 virus in the hosts including mice (Gabriel *et al*., 2008; Li *et al*., 2005).

Previous studies have shown that amino acid substitutions (e.g., residue 627 (E to K) and 701 (D to N) of PB2, 13 (L to P), 678 (S to N) of PB1, 614 (N to K) of PA and 319 (N to K) of NP) may increase the virulence of H5N1 virus in mammals (Gabriel *et al*., 2008; Li *et al*., 2005) in ostrich strain E627K of PB2 and L13P of PB1 only recorded.

Molecular characterization of M and NS genes: There are five amino acid sites that have been identified in the transmembrane region of the M2 protein are involved in resistance to the antiviral drug amantadine: sites L26F, V27A/S, A30 T, S31N and G34E (Suzuki *et al*., 2003). No amino acid Substitutions were found at these residues, suggesting that the Isolate are sensitive to this class of antiviral drugs.

NS1 protein plays an important role in the pathogenicity of H5N1 virus in different hosts. A five amino acid residue deletion at positions 80 to 84 was found in the NS1 molecule of the ostrich viruse as also characterized for the other H5N1 viruses of the Z+, Z, Y, A, B and C genotypes and this deletion may contribute to increased virulence (Long *et al*., 2008).

Previous studies showed that several mutations, such as P42S, D92E and V149A, may increase the virulence of H5N1 viruses in pigs, mice and chickens, respectively and P42S and V149A recorded in ostrich strain (Seo *et al*., 2004; Jiao *et al*., 2008).

Zhu *et al*. (2008) showed that a deletion of amino acids 191-195 of the NS1 protein could contribute to the attenuation of *Influenza A virus* in chickens (Zhu *et al*., 2008) This was not observed in ostrich H5N1 virus.

Large-scale sequence analysis of *Avian influenza* viruses indicated that the four C-terminal residues of the NS1 protein is a potential PDZ ligand binding motif of the X-S/T-X-V type (Obenauer *et al* 2006). The PDZ ligand binding motifs with the sequence of ESEV or EPEV were found in the NS1 from the highly pathogenic H5N1 viruses isolated in 1997 and 2003 as well as the 1918 pandemic virus (all of *Avian origin*). They are able to bind cellular PDZ-containing proteins involved in host cellular signaling pathways. In contrast, the NS1 protein in most low pathogenic human influenza viruses contain a different motif (RSKV or RSEV), which cannot bind PDZ-containing proteins. A recent study showed that the PDZ-binding motif of NS1 is a new virulence factor of influenza A viruses (Jackson *et al*., 2008). Ostrich virus possessed an "*Avian*-like" ESEV motif at the NS1 C-terminal region, PDZ domain motif, which may contribute to increased virulence.

DISCUSSION

HPAI H5N1 viruses become endemic in Egypt affecting all species of poultry including ostrich since it was firstly reported in 2006, The pathogenicity of H5N1 influenza virus is polygenic,

at least including HA, NA, PB2, PB1, PA, NP and NS genes (Peiris *et al*., 2007). Many studies indicate that pathogenicity depends on the functional integrity of each gene and on a cluster of genes that is optimal for infection (Gabriel *et al*., 2005, 2008; Salomon *et al*., 2006; Hulse *et al*., 2004). Recent studies have revealed that the high Pathogenicity of H5N1 virus is a complex phenotype dependent on both the virus and the host (Gabriel *et al*., 2005; Salomon *et al*., 2006).

HA plays an important role in determining the tissue tropism, systemic spread and pathogenicity of *Avian influenza* viruses. Influenza viruses attach to host cells by binding of the hemagglutinin to sialosaccharides on the host cell surface. Receptor binding preference is a major factor in determining host species tropism. Human influenza viruses prefer sialic acid (SA) -a-2, 6-Gal-terminated saccharides, whereas *Avian influenza* viruses prefer those terminating in SA -a-2, 3-Gal (Peiris *et al*., 2007).

In present study, the HA molecules of the ostrich H5N1 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, also contain a multibasic cleavage site in the HA. 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 Sialic Acid (SA) a 2, 6-Gal receptor (Zhu *et al*., 2008). These Mutations were not found in the HA of ostrich isolate, Implying that the isolates preferentially bind to a 2, 3-NeuAcGal Linkages of the *Avian* cell receptors rather than a2, 6-NeuAcGal Linkages of the human cell receptors, It was recorded that there is 6 amino acid residues (86V, 124S, L/N138, T/S156, E/R212, T263) that are linked to the virulence of H5N1 in mammals (Lycett *et al*., 2009) two of them (263T and T/S156) detected ostrich strain.

Also there is a deletion in amino acid No. 129 which is not found in H5N1 isolates from other endemic countries with H5N1 like Vietnam, Indonesia and Thailand while it present in most of human infections with H5N1 from 2009 till 2011 in Egypt and also present in all human seasonal H1N1 and H3N2 viruses (Abdelwhab *et al*., 2010).

A balance between the activities of HA in virus attachment and that of NA in virus release is crucial for optimal viral replication (Hulse *et al*., 2004). Similar to the other genotype V, the ostrich virus also exhibited a deletion in the stalk region of NA, which is proposed to be an adaptation of H5N1 viruses from aquatic birds to terrestrial poultry, such as chickens (Peiris *et al*., 2007).

Using reverse genetics, many studies revealed that some amino acid substitutions of PB2, PB1, PA and NS1 (ESEV motif) were associated with H5N1 pathogenicity in mammals (Hatta *et al*., 2001; Li *et al*., 2005; Hulse-Post *et al*., 2007).

Sequence analysis revealed amino acid residues P13 and Y436 in PB1 of ostrich H5N1 virus suggesting that the virus may be highly virulent in mice and ducks.

In present study ostrich virus acquire the K 627 amino acid substitution in PB2, Most *Avian* isolates have E in this position, The substitution to K in this position converts a nonlethal H5N1 *Influenza A virus* isolated from a human to a lethal virus in mice (Shinya *et al*., 2004). The virus also Contain amino acid residues P13 in PB1 and ESEV motif at the NS1 C-terminal region, PDZ domain motif, that may contribute to the pathogenicity in mice and ducks (Hulse-Post *et al*., 2007).

Also amino acid at position 701 in PB2 plays a crucial role in the ability of H5N1 viruses of duck origin to replicate and be lethal in mice (Li *et al*., 2005). This amino acid also contributes to the

increased lethality of an H7N7 AIV in a mouse model (Gabriel *et al*., 2005), PB2 residue 271 plays a role in the enhanced polymerase activity of *Influenza A* viruses in mammalian host cells (Bussey *et al*., 2010).

From the sequence data it is recorded that Ostrich strain sensitive to antiviral drugs as Amantadine and tamiflu.

REFERENCES

Abdelwhab, E.S.M., H.M. Hafez, M.M. Aly, C. Grund and T.C. Harder, 2010. Increasing prevalence of unique mutation patterns in H_5N_1 *Avian influenza* virus HA and NA Glycoproteins from human infections in Egypt. Sequencing, Vol. 2010. 10.1155/2010/450823

Alexander, D.J., 2000. A review of avian influenza in different bird species. Vet. Microbiol., 74: 3-13.

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.

Cattoli, G., M. Adelaide, N. Temperton, B. Zecchin and A. Buratin *et al*., 2011. Antigenic drift in H5N1 *Aavian influenza* virus in poultry is driven by mutations in major antigenic sites of the hemagglutinin molecule analogous to those for human influenza virus. J. Virol., 85: 8718-8724.

Chen, H., G.J. Smith, K.S. Li, J. Wang and X.H. Fan *et al*., 2006. Establishment of multiple sublineages of H_5N_1 *Influenza virus* in Asia: Implications for pandemic control. Proc. Natl. Acad. Sci. USA., 103: 2845-2850.

Gabriel, G., A. Herwig and H.D. Klenk, 2008. Interaction of polymerase subunit PB_2 and NP with importin a1 is a determinant of host range of *Influenza* A virus. PLoS Pathog, Vol. 4. 10.1371/journal.ppat.0040011

Gabriel, G., B. Dauber, T. Wolff, O. Planz, H.D. Klenk and J. Stech, 2005. The viral polymerase mediates adaptation of an *Avian influenza* virus to a mammalian host. Proc. Natl. Acad. Sci., 102: 18590-18595.

Hatta, M., P. Gao, P. Halfmann and Y. Kawaoka, 2001. Molecular basis for high virulence of Hong Kong H_5N_1 *Influenza* A viruses. Science, 293: 1840-1842.

Horimoto, T. and Y. Kawaoka, 1994. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J. Virol., 68: 3120-3128.

Huchzermeyer, F.W., 1998. Diseases of Ostriches and other Ratites. Onderstepoort Veterinary Institute, Pretoria, South Africa, Pages: 296.

Hulse, D.J., R.G. Webster, R.J. Russell and D.R. Perez, 2004. Molecular determinants within the surface proteins involved in the pathogenicity of H_5N_1 *Influenza viruses* in chickens. J. Virol., 78: 9954-9964.

Hulse-Post, D.J., J. Franks, K. Boyd, R. Salomon and E. Hoffmann *et al*., 2007. Molecular changes in the polymerase genes (PA and PB_1) associated with high pathogenicity of H_5N_1 *Influenza virus* in mallard ducks. J. Virol., 81: 8515-8524.

Jackson, D., M.J. Hossain, D. Hickman, D.R. Perez and R.A. Lamb, 2008. A new *Influenza virus* virulence determinant: The NS_1 protein four C-terminal residues modulate pathogenicity. Proc. Nat. Acad. Sci. USA., 105: 4381-4386.

Jiao, P., G. Tian, Y. Li, G. Deng and Y. Jiang *et al.*, 2008. A single-amino-acid substitution in the NS_1 protein changes the pathogenicity of H_5N_1 *Avian influenza* viruses in mice. J. Virol., 82: 1146-1154.

Le, Q.M., M. Kiso, K. Someya, Y.T. Sakai and T.H. Nguyen *et al.*, 2005. Avian flu: Isolation of drug-resistant H_5N_1 virus. Nature, Vol. 437. 10.1038/4371108a

Li, K.S., Y. Guan, J. Wang, G.J.D. Smith and K.M. Xu *et al.*, 2004. Genesis of a highly pathogenic and potentially pandemic H_5N_1 *Influenza virus* in eastern Asia. Nature, 430: 209-213.

Li, Z., H. Chen, P. Jiao, G. Deng and G. Tian *et al.*, 2005. Molecular basis of replication of duck H_5N_1 *Influenza viruses* in a mammalian mouse model. J. Virol., 79: 12058-12064.

Long, J.X., D.X. Peng, Y.L. Liu, Y.T. Wu and X.F. Liu, 2008. Virulence of H_5N_1 *Avian influenza* virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes, 36: 471-478.

Lycett, S.J., M.J. Ward, F.I. Lewis, A.F.Y. Poon, S.L.K. Pond and A.J.L. Brown, 2009. Detection of mammalian virulence determinants in highly pathogenic *Avian influenza* H_5N_1 viruses: Multivariate analysis of published data. J. Virol., 83: 9901-9910.

Munster, V.J., C. Baas, P. Lexmond, J. Waldenstrom and A. Wallensten *et al.*, 2007. Spatial temporal and species variation in prevalence of *Influenza* A viruses in wild migratory birds. PLoS Pathog., Vol. 3. 10.1371/journal.ppat.0030061

OIE, 2008. Update on highly pathogenic *Avian influenza* in animals (type H5 and H7). http://www.vets-net.com/Default.aspx?page=pages/news/NewsItem.aspx&query=QMitemEQ204

OIE, 2009. Update on highly pathogenic *Avian influenza* in animals (type H5 and H7). http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2009/.

Obenauer, J.C., J. Denson, P.K. Mehta, X. Su and S. Mukatira *et al.*, 2006. Large-scale sequence analysis of *Avian influenza* isolates. Science, 311: 1576-1580.

Peiris, J.S.M., M.D. de Jong and Y. Guan, 2007. *Avian influenza* virus (H_5N_1): A threat to human health. Clin. Microbiol. Rev., 20: 243-267.

Salomon, R., J. Franks, E.A. Govorkova, N.A. Ilyushina and H.L. Yen *et al.*, 2006. The polymerase complex genes contribute to the high virulence of the human H_5N_1 *Influenza virus* isolate A/Vietnam/1203/04. J. Exp. Med., 203: 689-697.

Senne, D.A., B. Panigrahy, Y. Kawaoka, J.E. Pearson and J. Suss *et al.*, 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: Amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis., 40: 425-437.

Seo, S.H., E. Hoffmann and R.G. Webster, 2004. RETRACTED: The NS_1 gene of H_5N_1 *Influenza viruses* circumvents the host anti-viral cytokine responses. Virus Res., 103: 107-113.

Shinya, K., S. Hamm, M. Hatta, H. Ito, T. Ito and Y. Kawaoka, 2004. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H_5N_1 *Influenza* A viruses in mice. Virology, 320: 258-266.

Spackman, E., D.A. Senne, T.J. Myers, L.L. Bulaga and L.P. Garber *et al.*, 2002. Development of a real-time reverse transcriptase PCR assay for type A *Influenza virus* and the Avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbiol., 40: 3256-3260.

Suzuki, H., R. Saito, H. Masuda, H. Oshitani and M. Sato *et al.*, 2003. Emergence of Amantadine-resistant *Influenza* A viruses: Epidemiological study. J. Infect. Che-Mother, 9: 195-200.

WHO/OIE/FAO H5N1 Evolution Working Group, 2009. Continuing progress towards unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: Divergence of clade 2?2 viruses. Influenza Other Respir. Viruses, 3: 59-62.

Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers and Y. Kawaoka, 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev., 56: 152-179.

Zhu, Q., H. Yang, W. Chen, W. Cao and G. Zhong *et al.*, 2008. A naturally occurring deletion in its NS gene contributes to the attenuation of an H_5N_1 swine *Influenza virus* in chickens. J. Virol., 82: 220-228.