Genetic and Pathogenic characterization of avian influenza H5N1 isolated from duck in Egypt

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Abstract: In this study Avian Influenza type A (H5N1) virus was isolated from duck farm eight weeks of age and showing clinical manifestation of avian influenza infection. The obtained isolate was differentiated from the haemagglutinating agents using different specific antisera. To confirm the pathogenicity of the isolated virus, Specific Pathogenic Free (SPF) chickens were infected with such isolate. The birds showed clinical manifestation and the total mortalities were 100% within 48 hours post-infection. The same results were obtained when the isolated virus was used in infection of ducks but with total mortalities 70%. Reverse transcriptase polymerase chain reaction (Rt-PCR) was conducted for both H5 and N1 genes using specific primers and the obtained PCR products were at the molecular weight of 219 and 616 bp respectively. Sequence analysis for both genes (H5 and N1) PCR products were analyzed then compared with such available on GenBank and the obtained results showed that 99% similarity with virus sequences of chicken origin for both H and N genes while it was the same result regarding H gene sequence of virus from duck origin yearly 2010 and 2011, while it was 98% and 97% similarity when compared with the N gene sequences yearling 2010 and 2011 respectively. The obtained results revealed that the AI virus isolated from ducks yearly 2010 showed great similarity with that causing epidemics yearly 2010 and 2011 and the virus was circulating between ducks and chickens which increases the epidemiology between farms in Egypt.
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Keywords: Avian Influenza H5N1, ducks, chicken, Egypt outbreaks, Genetic analysis, pathogenic characterization, sequencing, PCR of H gene, PCR of N gene.

1. Introduction

Avian influenza viruses belonged to Orthomyxoviridae family is represented as a major contributor to viral diseases in poultry as well as humans. Outbreaks of highly pathogenic avian influenza (HPAI) viruses cause high mortality in poultry, resulting in significant economic losses. It is thought that classically wild birds, especially waterfowl, act as reservoirs for AI viruses **(Kawaoka et al., 1988)**. Low-pathogenic avian influenza viruses (LPAI) appear to replicate preferentially in the gastrointestinal tract of wild ducks and other wild birds, resulting in the high-level excretion in faeces and the spread of infection via the faecal and oral route **(Hinshaw et al., 1980a)**. These viruses may be transmitted to poultry populations where in some cases mutation to HPAI viruses occurs **(Rohm et al., 1995; Banks et al., 2000)** .The highly pathogenic H5 subtypes of influenza viruses are highly pathogenic to chickens, quail, and pheasants **(Perdue et al., 1997).** The HA is the major antigen that elicits antibodies which protect against death and clinical signs **(Brugh and Stone, 1987)**. Virus representatives of all 15 haemagglutinin (HA) and all 9 neuraminidase (NA) subtypes have been isolated from waterfowl **(Webster et al., 1992).** Thus, ducks are considered as one of the primary reservoir for avian influenza viruses (AIV) and have been implicated in the spread of influenza to domestic poultry **(Halvorson et al, 1985)**. Recently, large-scale sequence analyses revealed 'signature' amino acids at specific positions in viral proteins that distinguish human influenza viruses from avian influenza viruses **(Chen et al., 2006 and Finkelstein et al., 2007)**. The present study was an attempt to isolate Avian influenza virus from infected duck farms and study its pathogenicity and sequencing analysis of the H5 and N1 genes.

2. Materials and Methods

Cloacal and tracheal samples were obtained from ducks related to duck farm showing clinical signs of influenza infection during the outbreaks of the avian influenza in Egypt yearly 2010 and causing mortality up to 62% between the flock. Swabs were soaked in 2ml of 2.95% tryptose phosphate buffer with 5×10^3 IU of penicillin-G-sodium and 5 mg streptomycin per ml. The samples were filtrated using a sterile membrane filter 0.45µ pore size, then a 0.2ml of the filtrate was inoculated into the allantoic cavity of five 9-day-old embryonated specific pathogen free (SPF) chicken eggs. Inoculated eggs were incubated at 37°C and examined daily for 72 hours. Allantoic fluids were harvested and tested for haemagglutination activity **(OIE, 2008).**

2- Antigenic analysis and virus titration.

The suspected samples were antigenically analyzed by HI test with a panel of antisera against H5, ND, EDS and IB hemagglutinin. HI assays were performed with microtiter plates as previously described by **(Palmer et al., 1975)**. Allantoic fluid was harvested and 50% egg infected dose (EID_{50}) titers were determined by testing the haemagglutination activity **(Swayne et al., 1998).** Titration endpoints were calculated by the method of Reed and Muench **(Reed and Muench, 1938**)

3- Pathogenicity studies in chickens

Allantoic fluid collected from the inoculated 9 day old SPF-ECE (embryonated chicken eggs) within 3 days post inoculations were used. Twenty four-week-old SPF chickens and another group of 8 weeks old chicken were inoculated intranasal with $10^{7.85}$ to $10^{8.5}$ EID_{50} of influenza virus in 0.1 ml and housed in special isolator. Inoculated birds were observed daily for clinical signs and mortality. Tracheal and cloacal swabs were collected daily post inoculation **(Sturm-Ramirez et al., 2004 & Guan et al., 1999)** and examined for virus re isolation. The same procedure was done using 8 weeks old ducks.

4- Viral RNA isolation and RT PCR.

Viral RNA was directly extracted from allantoic fluid of the inoculated SPF -ECE using RNA extraction kit (QIA amp viral RNA Mini-Qiuagen). The RNA was transcribed into cDNA then H5 and NA gene amplified using Bio RT-one step PCR kit (BioER). H5 and N1 gene primers were designed according to **(WHO Geneva. August 2007);** for H5 gene, H5-1: GCC ATT CCA CAA CAT ACA CCC and H5-3: CTC CCC TGC TCA TTG CTA TG. And for N1 gene, N1-1: TTG CTT GGT CGG CAA GTG C and N1-3: CCA GTC CAC CCA TTT GGA TCC. RT PCR mixture was prepared as: 2.5µl of 10x BioEr RT-PCR buffer, 4.0µl of dNTP mix, 2.0 µl of Forward primer (5 µM), 2.0 µl of Reverse primer (5 µM), 1.0 µl Taq polymerase enzyme, 0.5 µl RNase inhibitor (20U/ul), 1.0 µl AMV enzyme, 7.0µl of Molecular grade water and add 5µl viral RNA. PCR conditions were performed as: Reverse transcription 30 min 45º C followed by Initial PCR activation 4min 94ºC, 3 steps cycling of 35 cycling: Denaturation 30 sec 94ºC, Annealing 30 sec 58ºC, Extension 30 sec 72ºC and final extension 3 min 72ºC. Agarose gel was prepared 1.5%, loading of PCR product together with 100 bp molecular weight marker and run according to **Sambrook et al. (1989),** then visualized under UV light.

5- Sequencing and Phylogenic analysis of H5 and N1 influenza virus gene.

The nucleotide sequences of both amplicons either H5 or N1 genes were performed using the procedures described by **(Guan et al., 1999).** Sequencing were obtained using the big dye terminator sequencing kits for sequencing using an ABI-3700 sequencer (according to ABI system Big dye terminator version 3.1 and according to the manufacture's instruction). The sequencing information was complied with the segman program (DNASTAR, Madison). The obtained Sequences were aligned with the existing database using BLAST search tool available online (www.ncbi.nlm.nih.gov).

3. Results

1- Virus isolation and Identification

Avian influenza virus was isolated from duck samples obtained from 8 week old ducks showing clinical signs of influenza infection and causing a mortality exceed 62% between the flock. Postmortem lesions were noticed as petecheal hemorrhages the peritoneum, on abdominal and coronary fats. Also hemorrhages were found externally on the shank and muscles. Theses suspected samples were inoculated in the SPF-ECE which showing embryo mortalities within 24 hours post inoculation.

2-Results of the Hemagglutinin assay and HI Test

Haemagglutination agents were primary detected by haemagglutination of chicken RBCs then all hemagglutinating agents were tested in the HI test with anti-NDV, EDS, IB and AI serum. The HI test results were negative for antisera of all viruses except AI either H5N1. The virus titration was determined by (HI) test using antisera against H5N1 as 10^5 log_2 for the primary isolates by using antisera against H5N1.

3- Result of Virus Pathogenicity.

The inoculated SPF white Leghorn chicken showed clinical signs of avian influenza and deaths within two days post inoculation and the mortality was 100%. Regarding the pathogenicity in the ducks was the same as in chicken but the mortality were only up to 70% as shown in table (1). The AI virus was re-isolated from tracheal and cloacal swabs collected daily post inoculation either from chickens or ducks.

Table (1): Results of Pathogenicity test of isolated Avian Influenza from duck in Egypt yearly 2010.

Host	Number used	Mortality		Virus re-isolate	
		deaths	Percentage	Recovery	Percentage
Chicken	20	20/20	100	20/20	100
Duck	20	14/20	70	20/20	100

4- AI Virus Characterization.

AI Viral RNA was detected by the RT-PCR using the specific primers of either H5 or N1 genes. The specific visible bands were visualized and measured with the molecular size of 219 bp and 616 bp for both H5 and N1 gene respectively as shown in photos (1 and 2). The obtained PCR products were sequenced using soft ware for both genes and compared to sequences available in Gene Bank as shown in tables (2 and 3) and fig (1 and 2) with over all alignment around 99%. The obtained sequences were compared with either sequence available for chicken H5 yearling 2010 Influenza A virus [A/chicken/Egypt/10159s/2010(H5N1)] segment 4 hemagglutinin (HA) gene, complete cds) and 2011(Influenza A virus [A/chicken/Egypt/113Q/2011(H5N1)] segment 4 hemagglutinin (HA) gene, complete cds) and for the gene N1 yearling 2010 (Influenza A virus [A/chicken/Egypt/1042/2010(H5N1)] segment 6 neuraminidase (NA) gene, partial cds) and 2011 (Influenza A virus [A/chicken/Egypt/116AD/2011(H5N1)] segment 6 neuraminidase (NA) gene, partial cds) and the alignments were significant up to 99% with all as shown in fig. (3, 4, 5 and 6). While on comparing the isolates with duck isolates available on GenBank for the H5 yearling 2010 (nfluenza A virus (A/duck /Egypt /106d /2010 (H5N1)) segment 4 hemagglutinin (HA) gene, complete cds) and 2011 [Influenza A virus (A/ duck/ Egypt/ 1123SF/ 2011 (H5N1)] segment 4 hemagglutinin (HA) gene, complete cds) , the alignments were significant up to 99% with H5 sequences as shown in fig (7and 8) while in case of N1 gene alignment were 98% and 97% yearling 2010 and 2011 respectively as shown in fig. (9 and 10). Regarding sequencing analyses comparing with field isolates of ducks yearly 2010 were 99% for both genes and were 99% yearly 2011 for both genes as shown in fig (3 and 4).

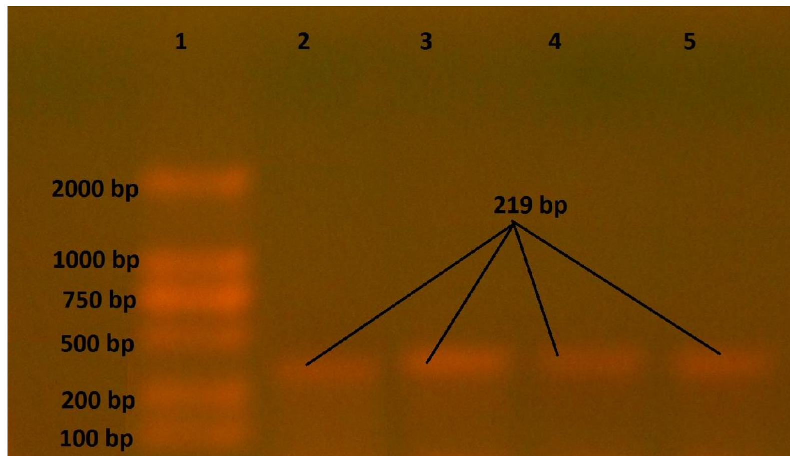


Photo (1);PCR amplicons of H5 gene of duck H5N1 virus isolated from infected ducks farm yearly 2010.

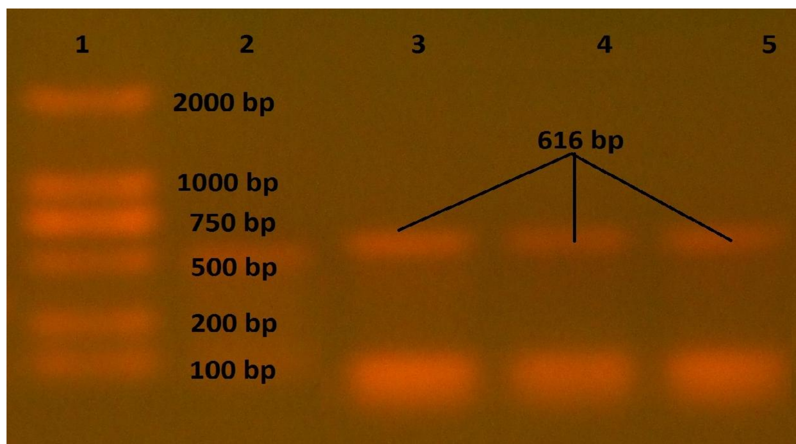


Photo (2);PCR amplicons of N1 gene of duck H5N1 virus isolated from infected ducks farm yearly 2010.

Table (2) Sequences producing significant alignments with the filed isolate of avian influenza H5N1 [A/Duck/Egypt/ 2010(H5N1)].

Accession	Description	Total score	Query coverage	E value	Max indent
FR687255.1	Influenza A virus (A/chicken/Egypt/1/2009(H5N1)) segment 4,	303	100%	2e-79	99%
JN807777.1	Influenza A virus (A/chicken/Egypt/10158SF/2010(H5N1))	303	100%	2e-79	99%
JN714467.1	Influenza A virus (A/chicken/Egypt/VSVRI/2009(H5N1))	303	100%	2e-79	99%
JF357723.1	Influenza A virus (A/chicken/Egypt/F10/2009(H5N1)) segment	303	100%	2e-79	99%
HQ198296.2	Influenza A virus (A/chicken/Egypt/10265s/2010(H5N1))	303	100%	2e-79	99%
HQ198295.2	Influenza A virus (A/chicken/Egypt/10159s/2010(H5N1))	303	100%	2e-79	99%
HQ198281.2	Influenza A virus (A/chicken/Egypt/10116s/2010(H5N1))	303	100%	2e-79	99%
HM466695.1	Influenza A virus (A/chicken/Israel/65/2010(H5N1)) segment	303	100%	2e-79	99%
GU002686.1	Influenza A virus (A/duck/Egypt/09224F-NLQP/2009(H5N1))	303	100%	2e-79	99%
GU002678.1	Influenza A virus (A/duck/Egypt/0955-NLQP/2009(H5N1))	303	100%	2e-79	99%
FJ785148.1	Influenza A virus (A/domestic	303	100%	2e-79	99%
FJ785147.1	Influenza A virus (A/mute	303	100%	2e-79	99%
JQ520362.1	Influenza A virus (A/chicken/Turkey/Enez	298	100%	8e-78	99%
JQ520357.1	Influenza A virus (A/chicken/Turkey/Esetce	298	100%	8e-78	99%
FR687256.1	Influenza A virus (A/chicken/Egypt/Q1011/2010(H5N1))	298	100%	8e-78	99%
JN807816.1	Influenza A virus (A/duck/Egypt/1123SF/2011(H5N1))	298	100%	8e-78	99%

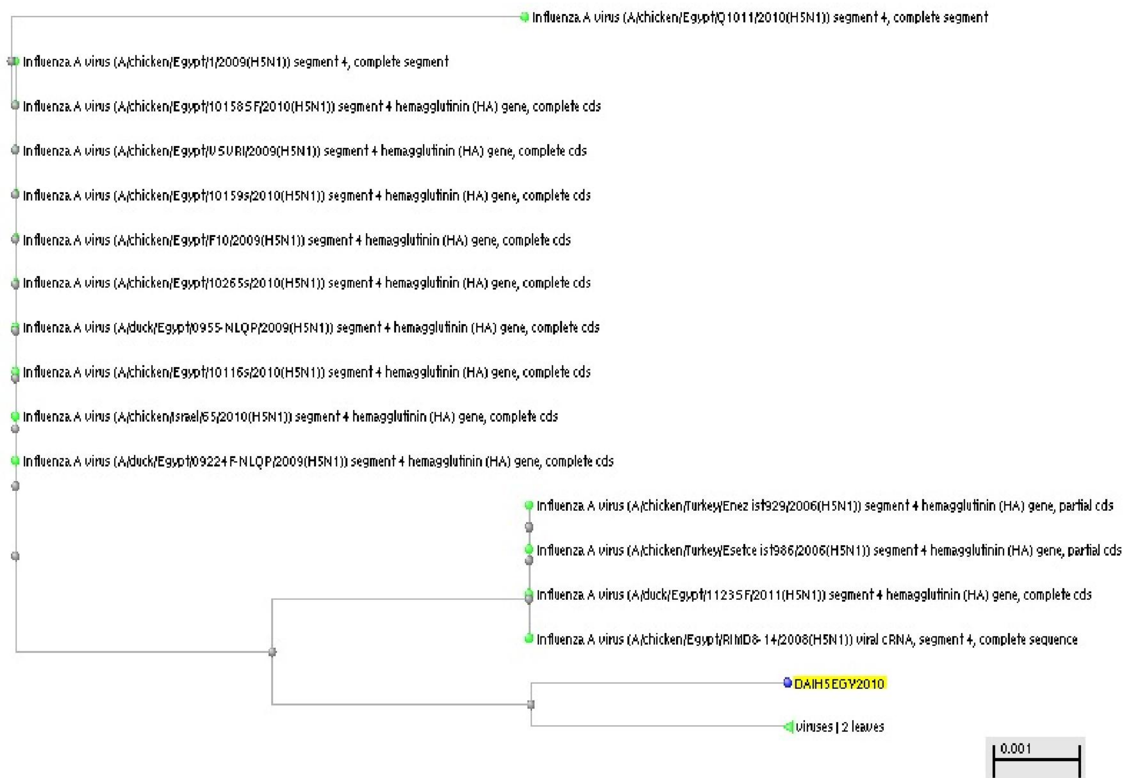


Fig.(1);Phylogenic analysis of the H5 gene of AI isolates from ducks compared with AI sequences obtained from Gene Bank database

Table (3) Sequences producing significant alignments with the filed isolate of avian influenza H5N1 segment 6 neuraminidase (NA) gene [A/Duck/Egypt/ 2010((H5N1)].

Accession	Description	Total score	Query coverage	E value	Max indent
HQ908470.1	Influenza A virus (A/chicken/Egypt/09534S-NLQP/2009(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	1024	96%	0.0	99%
HQ908466.1	Influenza A virus (A/chicken/Egypt/0918Q-NLQP/2009(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	1024	96%	0.0	99%
JN582042.1	Influenza A virus (A/turkey/Israel/362/2011(H5N1)) segment 6 neuraminidase (NA) gene, complete cds	1018	96%	0.0	99%
HQ908477.1	Influenza A virus (A/chicken/Egypt/1042/2010(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	1018	96%	0.0	99%
HQ908464.1	Influenza A virus (A/duck/Egypt/09224F-NLQP/2009(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	1018	96%	0.0	99%
GQ184286.1	Influenza A virus (A/chicken/Egypt/08139S-NLQP/2008(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	1018	96%	0.0	99%
AB601140.1	Influenza A virus (A/chicken/Egypt/RIMD4-5/2008(H5N1)) viral cRNA, segment 6, complete sequence	1013	96%	0.0	99%
HQ908485.1	Influenza A virus (A/chicken/Egypt/10127s/2010(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	1013	96%	0.0	99%
EU574921.1	Influenza A virus (A/chicken/Israel/1055/2008(H5N1)) neuraminidase (NA) gene, complete cds	1013	96%	0.0	99%
HQ908478.1	Influenza A virus (A/chicken/Egypt/1055/2010(H5N1)) segment 6 neuraminidase (NA) gene, partial cds	996	96%	0.0	99%
GQ355843.1	Influenza A virus (A/duck/Wels/2025/2006(H5N1)) neuraminidase (NA) gene, partial cds	996	96%	0.0	99%
EU152220.1	Influenza A virus (A/tufted duck/Switzerland/V504/2006(H5N1)) neuraminidase (NA) gene, complete cds	996	96%	0.0	99%
EU152218.1	Influenza A virus (A/duck/Switzerland/V426/2006(H5N1)) neuraminidase (NA) gene, complete cds	996	96%	0.0	99%
EU152217.1	Influenza A virus (A/duck/Switzerland/V389/2006(H5N1)) neuraminidase (NA) gene, complete cds	996	96%	0.0	99%
CY021391.1	Influenza A virus (A/chicken/Sudan/2115-10/2006(H5N1)) segment 6, complete sequence	996	96%	0.0	99%

Fig. (2) Phylogenic analysis of the N1 gene of AI isolates from ducks compared with AI sequences obtained from Gene

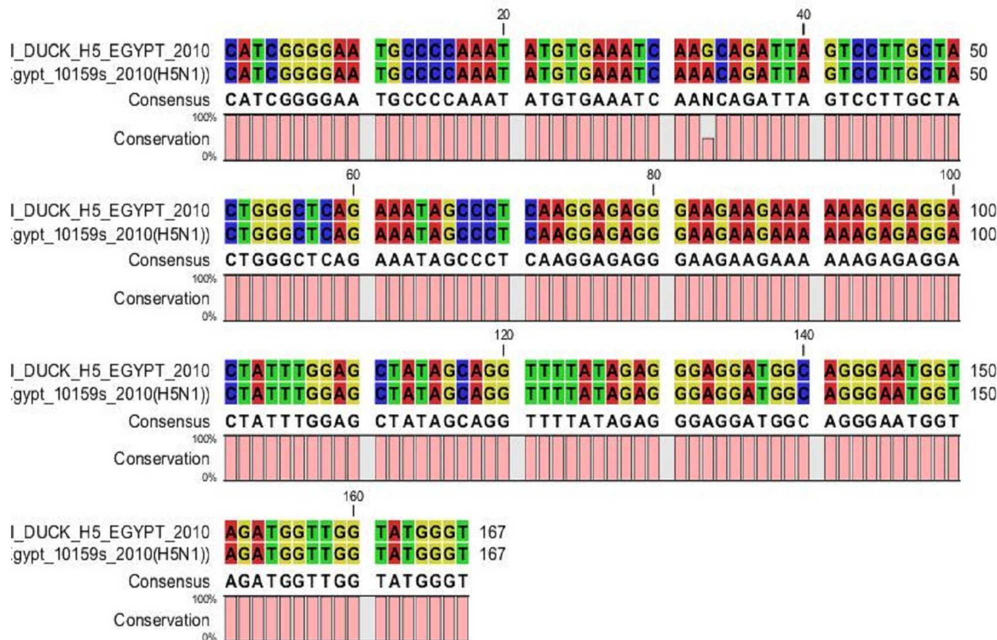


Fig.(3):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck /2010(H5N1- sample) and the published sequence of the H5 gene (A-Chicken/Egypt/10159s-2010-H5N1) in gene bank data base.

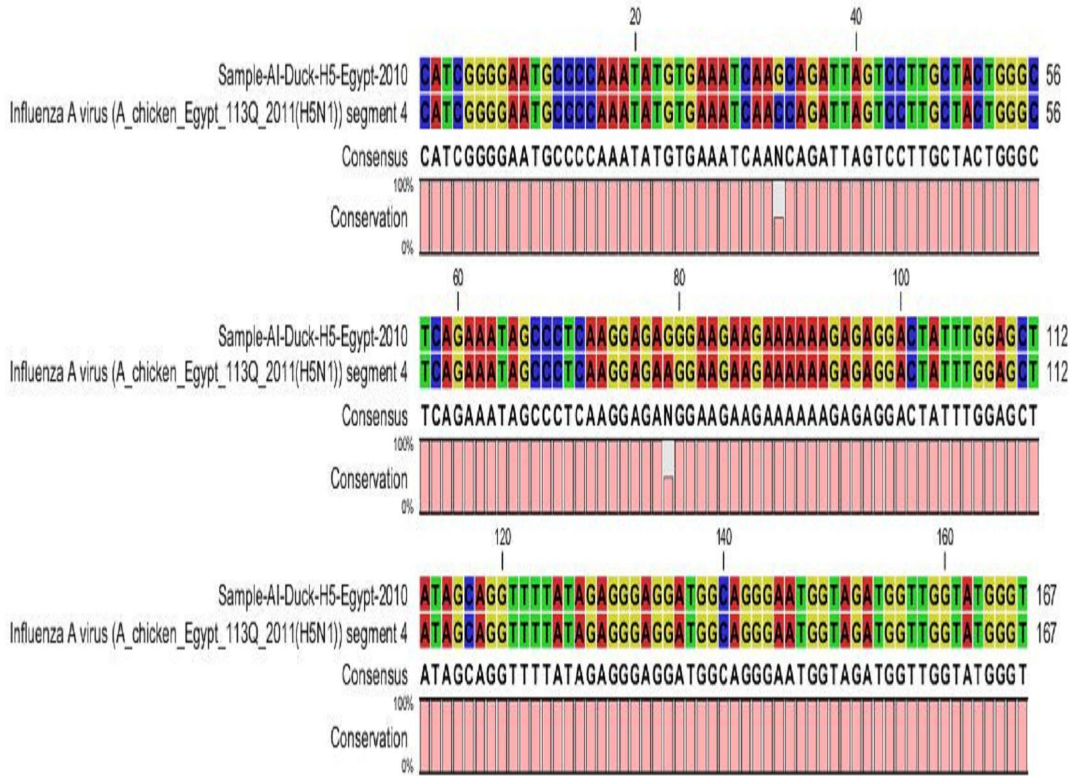


Fig.(4):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck//2010(H5N1)-sample) and the published sequence of the H5 gene (A-Chicken/Egypt/113Q-2011-H5N1) in gene bank data base.

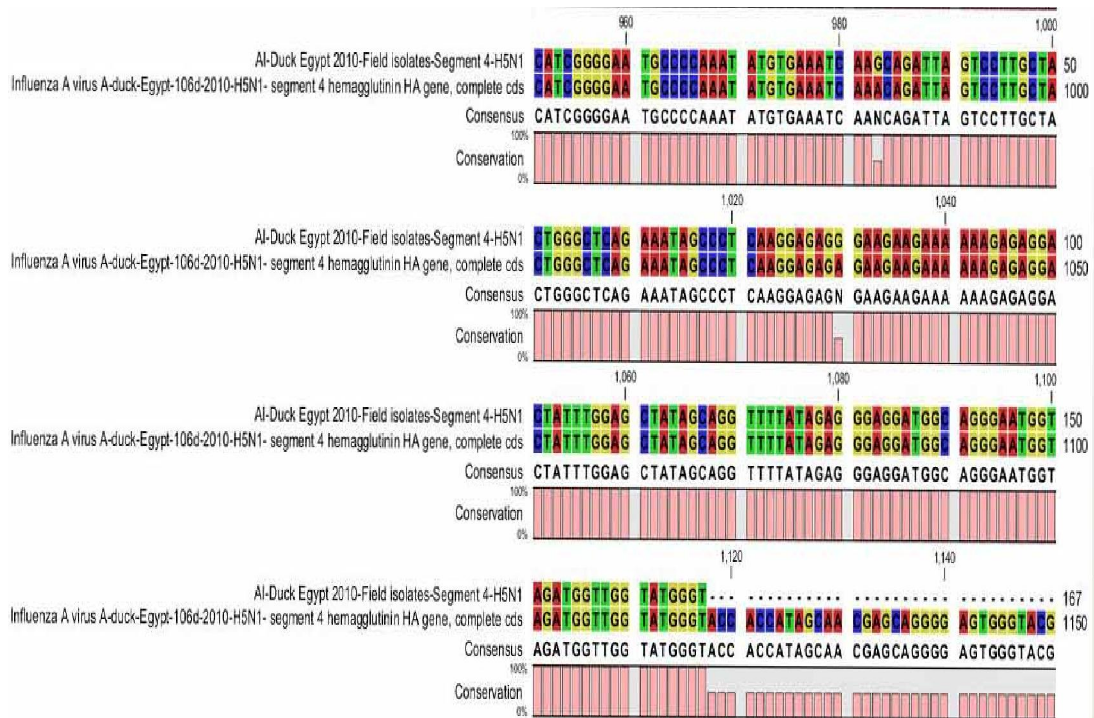
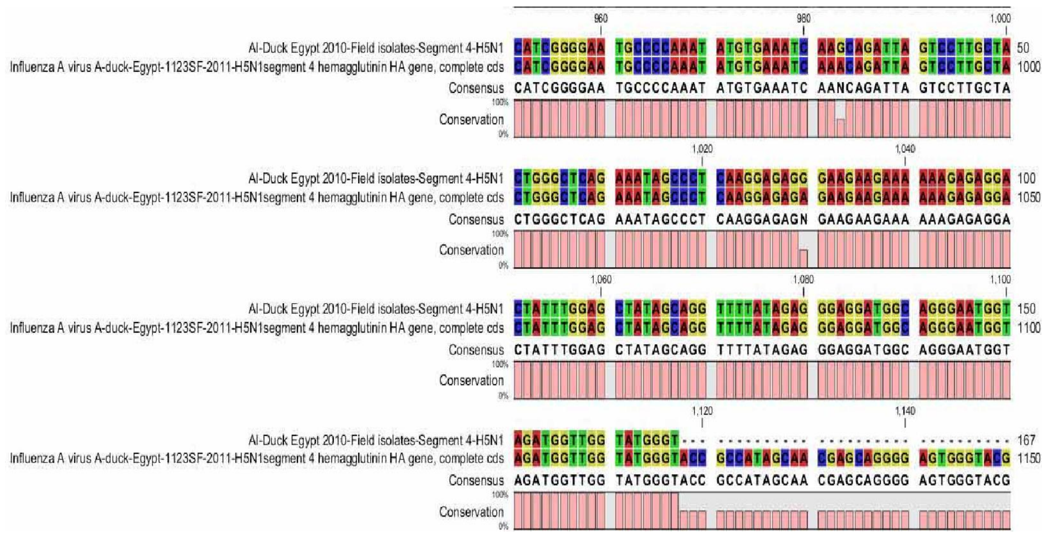


Fig.(5):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck/Egypt/2010(H5N1 sample) and the published sequence of the N1 gene (A_Chicken/Egypt/1042-2010-H5N1) in gene bank data base.



Fig(6):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck/Egypt/2010(H5N1 sample) and the published sequence of the N1 gene (A-Chicken/Egypt/116AD-2011-H5N1) in gene bank data base.

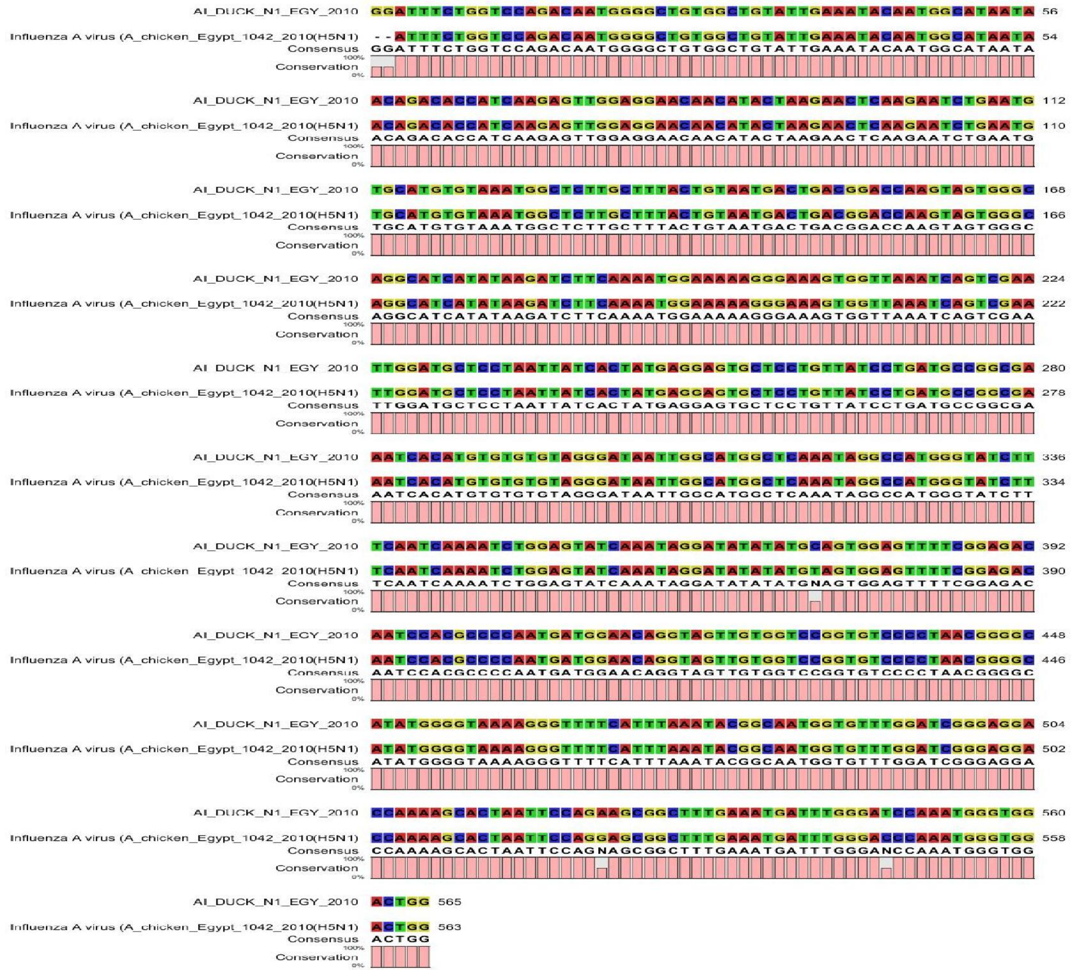
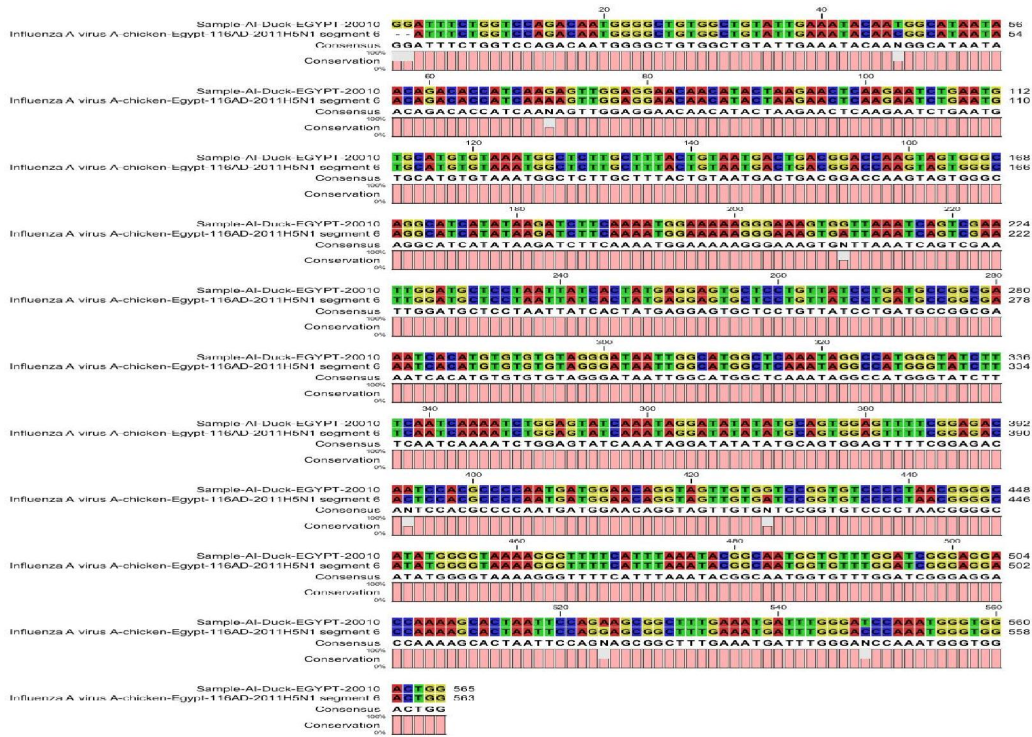


Fig.(7):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck/Egypt/2010(H5N1 sample) and the published sequence of the N1 gene (A_Chicken/Egypt/1042-2010-H5N1) in gene bank data base.



Fig(8):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck/Egypt/2010(H5N1 sample) and the published sequence of the N1 gene (A-Chicken/Egypt/116AD-2011-H5N1) in gene bank data base.

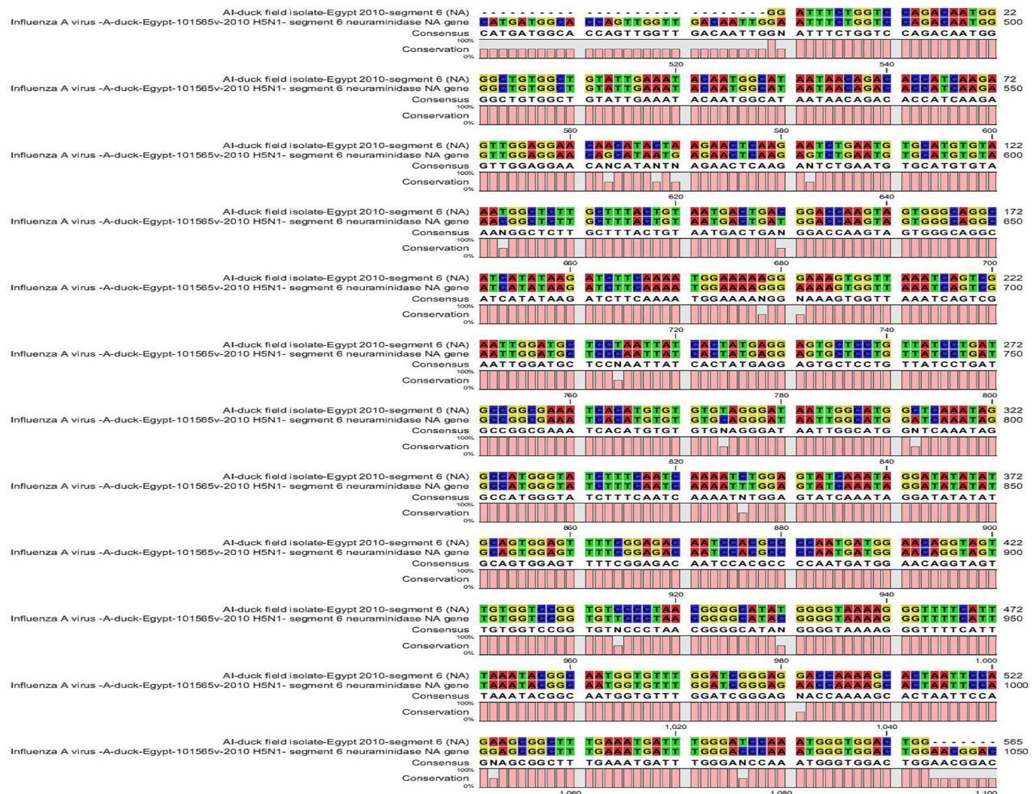


Fig.(9):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck/Egypt/2010(H5N1 sample) and the published sequence of the N1 gene (A-Duck/Egypt/101565v-2010-H5N1) in gene bank data base.

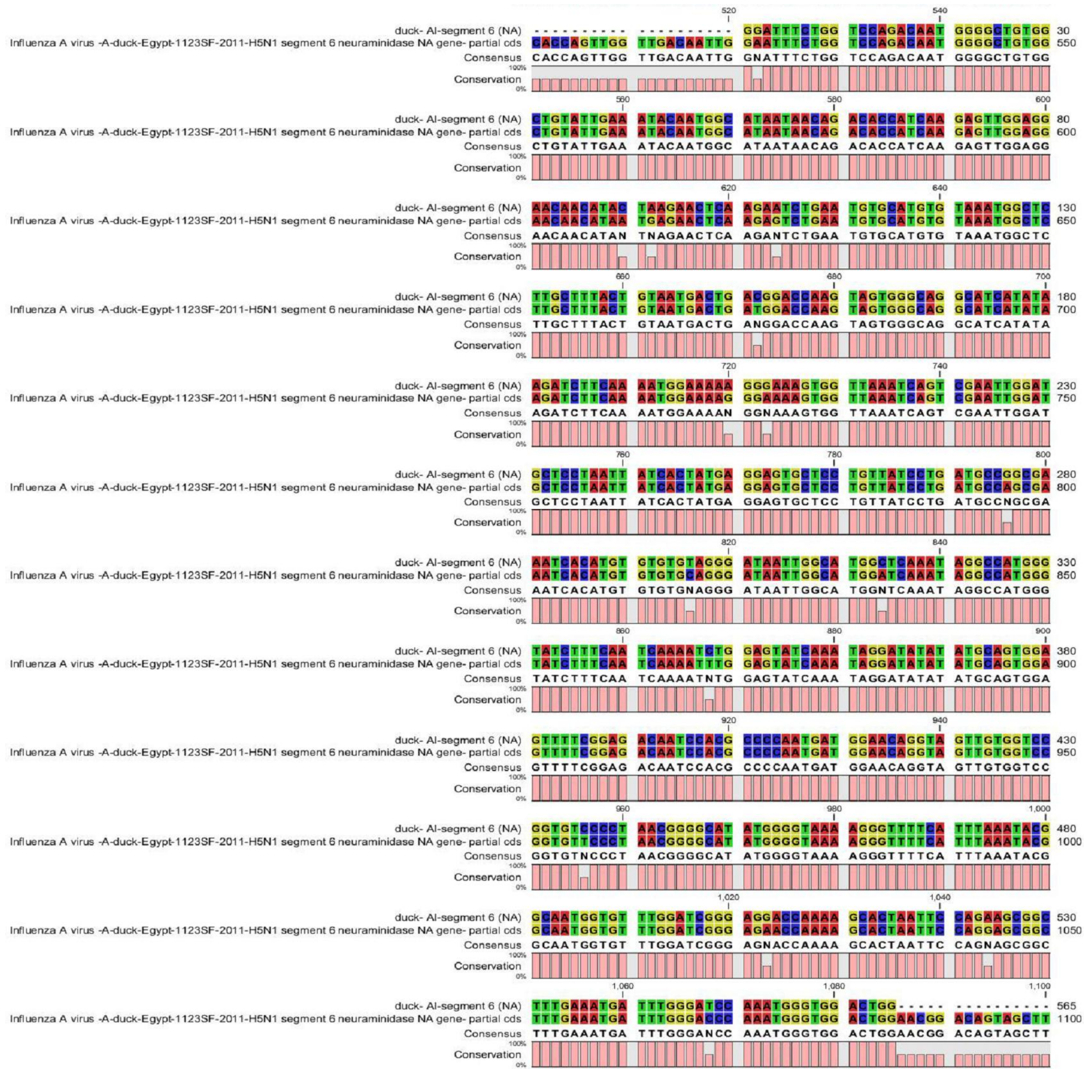


Fig.(10):Nucleotide sequence alignment between the sequence of the field isolate (A/Duck/Egypt/2010(H5N1 sample) and the published sequence of the N1 gene (A-Duck/Egypt/1123SF-2011) in gene bank data base.

4. Discussion

Historically, ducks have been considered to be clinically resistant to HPAI viruses and experimentally such viruses have not caused clinical disease or mortalities. Even though virus have been shown to spread systemically in experimentally infected ducks (**wood et al., 1995**). In contrast, in this study and during winter of 2010 in Egypt, avian influenza was isolated from duck showing clinical sign of avian influenza and the mortalities exceed 62% between flocks. **Brandon et al., (2008)** reported that H5N1 infected Pekin ducks 4 days post infection showed clinical signs as depression, reluctance to feed, in-coordination and torticollis while at 5 days post infection, the condition deteriorated rapidly and ducks appeared severely sick and thought unlikely to survive the infection. Haemagglutination inhibition test using specific antisera against H5 revealed that the isolated virus was H5N1. The virus was tested for purity using mono specific antisera. **Kosuke et al.,(2008)** stated that the antigenic analysis of H5 influenza viruses to the HA molecule confirmed that H5 isolates from water birds and the HPAI viruses share epitopes at the globular heads of the HAs although there were some differences in reactivity with mono clonal antibodies (MABs). **Slemones et al., 1973**, tested all haemagglutinating agent in the haemagglutination test with anti NDV serum and attempted to detect type A infected virus by means of agar gel diffusion test. Pathogenicity of the isolated viruses from duck when infected in SPF white Leghorn chicken showed clinical signs of avian infection and 100% mortalities, while in ducks showed clinical signs with only 70% mortalities. **Kishida et al., (2005)** reported that infected ducks with H5 influenza virus showed neurological signs

with blindness. Also virus was recovered from multiple organ of the bird. Also, **Tumpey et al.,2003**, demonstrated that, avian influenza virus isolated from duck meat caused 100% mortality of young chickens and disease signs observed were typical of those seen in chicken infected with H5 N1. Gross lesions observed induced sever pulmonary edema ,necrosis of the comb and edema of the brain , necrosis and the edema of the brain and the main death time were 2-3 days following inoculation. Regarding the Rt-PCR results, the specific visible bands were measured at the molecular weight of 219 bp and 616 bp for both H5 and N1 genes as noticed in Photo (1&2). These results were confirmed by that obtained by **Taha et. al., (2006)**, who proved the presence of H5N1 in the isolates by Rt-PCR and the obtained amplicon were 219 and 616bp for H5 and N1 subtypes respectively. Comparison of the nucleotide sequence of other HA or N1 gene for Rt-PCR products were further characterized by nucleotide sequencing and alignment with those sequences available of infected viruses on Gene Bank yearly 2010 and 2011 as shown in table (2 &3) and also shown in fig.(1&3) for genes H5 and N1 respectively. The results of isolated H5N1 represented typical HPAI viruses and the similarity were up to 99% between nucleotide sequences of isolated strain and the available published strain on Gene Bank yearly 2010 and 2011 for the H5 gene while N1 was 99% with the chicken isolates 2010 and 2011 and was 97% and 98% with ducks isolates 2010 and 2011 respectively. This means that these isolated AI strains were circulating isolates between chicken flocks and ducks flocks in Egypt. These results were proved by that obtained by **Taha et al., 2006**. **Kosuke. et. al., (2008)**, mentioned that the HA genes of the H5 isolates were sequenced and analysed along with those of other H5 strain , including HPAI viruses presently circulating in Asia and the amino acid sequenced cleavage site of the HA was deduced from the nucleotide sequence of the corresponding gene of each of the isolates. Meanwhile, **Tumpey et al., (2003)** stated that, the HA gene had the highest sequence similarity 98.4% with the 1999 Hong Kong isolates. In conclusion, The HPAI virus isolated from ducks yearly 2010 was clearly similar to that causes outbreaks of in chicken yearly 2010 and 2011 and such isolates currently circulating between ducks and chicken flocks resulting in possible recurrent outbreaks specially between chickens flocks because of domestic was considered a source of virus shedding and distribution between farms.

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