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List of Abbreviations

A.A	Amino Acids
Ag	Antigen
AGPT	Agar gel precipitation test
AI	Avian influenza
AIV	Avian influenza virus
bp	Base pair
BLAST	Basic Local Alignment Search Tool
Cat#	Catalogue number
CDC	Centers for Disease Control
cDNA	Complementary Deoxyribo Nucleic acid
C&D	Cleaning &Disinfection
CLEVB	Central Laboratory for Evaluation of Veterinary Biologicals
CT	Cycle Threshold
DDW	double distilled water
ECE	Embryonated chicken egg
EID	Egg Infectious Dose
ELISA	Enzyme Linked Immunosorbent assay
EMPRES	Emergency Prevention System for Animal Health
FAO	Food and Agriculture Organization
G	Gram
GLEWS	The Global Early Warning System
GOVS	General Organisation of Veterinary Services
HA	Haemagglutinin
HI	Haemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
HPNAI	Highly Pathogenic Notifiable Avian Influenza
HAU	Haemagglutination unites
IFT	Immunofluorescence test
IFN	Interferon

IRD	**Influenza Research Database**
LBMs	**Live Bird Markets**
LPAI	**Low Pathogenic Avian Influenza**
LPNAI	**Low Pathogenic Notifiable Avian Influenza**
M	**Matrix protein**
Mg/ml	**Milligram/ milliliter**
µl	**Microliters**
MID	**Minimum Infectious Dose**
MEGA	**Molecular Evolutionary Genetics Analysis**
mRNA	**Messenger RNA**
NA	**Neuraminidase**
NCBI	**National Center For Biotechnology Information**
NP	**Nucleoprotein**
NS	**Non-Structural protein**
NDV	**New Castle disease virus**
OIE	**Office International des Epizooties**
PA	**Polymerase acidic**
PB	**Polymerase basic**
PBGS	**Phosphate Buffered Gelatin Saline**
PBS	**Phosphate Buffer Saline**
Pcs	**Proteolytic Cleavage site**
RECP	**Restriction Enzyme Cleavage Pattern**
RNAs	**Ribonucleic acids**
RNP	**Ribo-Nucleoprotein**
rRT-PCR	**Real time reverse transcription polymerase chain reaction**
rpm	**Round per minute**
RT	**Room Temperature**
RT-PCR	**Reverse Transcription –Polymerase Chain Reaction**
Sec	**Second**
SPF	**Specific Pathogen Free**
TCID	**Tissue Culture Infectious Dose**
VI	**Viral Isolation**

VNT	Viral Neutralization Test
WHO	World Health Organization
Amino Acid	Single/Three Letter Amino Acid Code
Alanine	A Ala
Arginine	R Arg
Asparagine	N Asn
Aspartic Acid	D Asp
Glutamine	Q Gln
Glutamic Acid	E Glu
Glycine	G Gly
Isoleucine	I Ile
Leucine	L Leu
Lycine	K Lys
Methionine	M Met
Phenylalanine	F Phe
Proline	P Pro
Serine	S Ser
Threonine	Th Thr
Tryptophan	T Trp
Valine	V Val

6. Summary

Avian influenza (AI) represents one of the greatest concerns for public health that has emerged from the animal reservoir in recent times. AI, in its highly pathogenic form (HPAI-H5N1) has disseminated widely across Asia, Europe, and Africa, infecting a wide range of domestic and wild avian species and sporadically infecting humans and other mammals since its emergence in 1996 in Guangdong, China.

This thesis aimed to study the molecular characteristics of AIVs in gallinaceous birds in Upper Egypt during the year of 2014 and 2015. A total no. of 50 tracheal swab samples were collected from gallinaceous birds (chickens and turkeys) from the backyard and farms (which had a history of respiratory tract affections and high mortality rate. The collected swabs were tested with avian influenza rapid antigen test kit which is a chromatographic immunoassay for rapid detection or typing of avian influenza virus. Among 50 examined samples we had 8 positive ones with 16% positive rate .

The collected swab samples were examined with Real Time Reverse Transcriptase-PCR (rRT-PCR) technique for molecular subtyping using specific primer and probe designed for H5. rRT-PCR reactions were carried out by using Qiagen one step rRT-PCR kit with a certain PCR condition and results revealed that 48/50 were positive for H5 subtype (34 chicken samples and 14 turkey samples) (96% positive rate).

Virus isolation was done by inoculation of embryonated chicken eggs (9-11) day via allantoic sac route; the harvested allantoic fluid collected from dead embryos or at the end of (2-5) days post inoculation was tested by rapid and slow haemagglutination tests. Haemorrhages on the allantoic sac, severe embryonic congestion and haemorraghic and congested

chorioallantoic membrane were observed. The allantoic fluid was positive for rapid slide HA test (sandy appearance due to RBCs aggregates). H5 positive samples by rapid HA were undego further examination by micro haemagglutination test where the titer ranged from 1:64 to 1:256 (HAU varied from $\log_2 6$-8) according to the concentration of the virus in the collected allantoic fluid.

Molecular characterization of the virus was done by performance of two step RT-PCR for the extracted viral RNA. PCR amplification and sequencing were performed using degenerative primers for obtaining 545bp. Samples subjected to two step RT-PCR gave the desired PCR product. Gel extraction was done for further purification.

The purified PCR product of 5isolates were selected for partial haemagglutinin gene sequencing and phylogenetic analysis.

The nucleotide sequence analysis revealed that our isolates showed greatest nucleotide identities with 2017 Egyptian H5N1 AIV isolates which ranged from 95.8 to 98.6 % while the homology ranged from 93.8 to 98.2 % with 2015, from 93.1 to 97.6 % with 2014, from 86.4 to 97.4% with 2016, from 43.1-96.5% with 2013, 94.3 to 96% with 2012 and 29.4-41.7% with 2011respectively.

Nucleotide analysis of (A-Chicken-Egypt-Assiut2-2017 H5) strain revealed that this strain was far from the previously characterized Egyptian H5N1 AIV isolates which ranged from 46.2 to 48.1% with 2015, from 47.1 to 47.9% with 2017, from 45 to 47.5% with 2014, from 44.4 to 47.3% with 2016, from 45.4 to 46.7% with 2012, from 36.5 to 41% with 2013 and from 28.9 to 34.4% with 2011.

Comparing the HA gene of the isolates with the HA gene of some vaccinal strains commonly used in Egypt (A/Duck/Anhui/1/06, A/duck/Potsdam/1402/86, A/chicken/Mexico/232/94); the analysis

showed that the examined isolates were genetically distant from the vaccinal strains commonly used in Egypt which is lowest with A/chicken/Mexico/232/94 vaccinal strains in a percentage ranged from 76.8 to77.3% with four of our isolates and in a percentage of 44.6% with (A-Chicken-Egypt-Assiut2-2017 H5) Isolate that was the most distant.

The analysis results of the deduced amino acids of the haemagglutinin (HA) gene cleavage site for the obtained H5 sequences showed that all the isolates had QRERRRKKRG motif at the (PCS) confirming the highly pathogenic nature of the H5 isolates.

Phylogenetic analysis based on the HA partial nucleotide sequence of the isolates showed that four of our isolated strains Chicken-Egypt-Assiut1-2017 H5), (A-Chicken-Egypt-Assiut3-2017 H5), (A-Chicken-Egypt-Assiut4-2017 H5), (A-Chicken-Egypt-Assiut5-2017 H5) were clustered with the endemic subclade 2.2.1.2 and only one isolate (A-Chicken-Egypt-Assiut2-2017 H5) was clustered with distinct subclade 2.5, which resemble to (DQ992778/A/goose/ Shantou/239/2006/H5N1/2.5) and (DQ997276/A/ goose/Jilin/hb/ 2003/ H5N1/2.5) that belong to subclade 2.5.

Keywords: avian influenza virus; Real Time Reverse Transcriptase-PCR; partial haemagglutinin sequencing; Nucleotide analysis; phylogenetic analysis.

7. Conclusion

The results of this study concluded that:

- Avian influenza virus was responsible for signficant losses in poultry industry and potential zoonotic importance.
- Subtyping of isolates occurred via rRT-PCR with the use of specific primer and probe indicate the presence of H5 subtype of AIV in the area of study.
- Avian influenza virus led to pathological lesions in embryonated chicken eggs after allantoic sac route inoculation.
- Our isolates showed the highest similarity percentages were with 2017, 2015, 2014, 2016, 2013, 2012 Egyptian H5N1 AIV isolates respectively and the lowest homology was with 2011, which confirm that the AIVs continue to mutate and rapidly evolve over time.
- The analysis results of the deduced amino acids of the haemagglutinin (HA) gene cleavage site for the obtained H5 sequences showed that all the five isolates harbored highly pathogenic characteristics such as multiple basic amino acids at the HA polybasic cleavage site (PCS) (QRERRRKKRG) motif.
- Four of our isolated strains A-Chicken-Egypt-Assiut1-2017 H5), (A-Chicken-Egypt-Assiut3-2017 H5), (A-Chicken-Egypt-Assiut4-2017 H5), (A-Chicken-Egypt-Assiut5-2017 H5) were clustered with the endemic subclade 2.2.1.2 and only one isolate (A-Chicken-Egypt-Assiut2-2017 H5) was clustered with distinct subclade 2.5, and this isolate was close to (DQ992778/A/goose/Shantou/239/2006/H5N1/2.5) and (DQ997276/A/goose/Jilin/hb/ 2003/ H5N1/2.5) that belong to subclade 2.5.

- The HA gene of the examined H5 isolates especially (A-Chicken-Egypt-Assiut2-2017 H5) isolate were genetically distant from the vaccinal strains commonly used in Egypt which was 88.9 to 89.4% with A/Duck/Anhui/1/06 strain (the Chinese re-assorted vaccine, inactivated Re-5/H5N1) with four of our isolates, in a percentage of 44.8% with (A-Chicken-Egypt-Assiut2-2017 H5) isolate and was lowest with A/chicken/Mexico/232/94 vaccinal strain (Mexico H5N2 AI vaccine) in a percentage ranged from 76.8 to77.3% with four of our isolates and in a percentage of 44.6% with (A-Chicken-Egypt-Assiut2-2017 H5) isolate. This may suggest that efficacy of vaccines designed to control these viruses in poultry will reduce. Therefore, it may be necessary to evaluate the effectiveness of the current vaccine used in Egypt against circulating strains.
- Our findings raise the concerns about the role of using influenza vaccines in correlation with the development of antigenic drift in influenza epidemics

Recommendations:

-Molecular studies need to be sustained to identify the viral genetic changes that lead to variations of Influenza viruses in the field.

-Matching vaccine strains to currently circulating strains.

-Maintaining high vaccination coverage.

-The commercial vaccines need to be evaluated annually for evaluation of the vaccine efficacy in the face of antigenic drift.

-Vaccination must be accompanied by other control measures, including strict biosecurity measures, quarantines, controlled depopulation and increased surveillance.