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The Evolution and Molecular Characteristics of Polymerase Complex of H9N2 Avian Influenza Viruses in Egypt

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ABSTRACT

H9N2 avian influenza virus is endemic in Egyptian domesticated birds since 2010.Due to their mammalian-like traits, H9N2 have crossed the species barrier resulting in mild to moderate infections. Currently, the H9N2 virus's capacity to contribute their genes to other AIVs, creating high and low pathogenic IAVs that might cross species barriers and infect people, is considered a global concern. It has been determined that the RNA-dependent RNA polymerase of viruses (vRdRp) (polymerase complex) activity possesses a crucial role in modulating AIVs host adaptability to mammals and controlling of the viral ability to cross species barrier. Our study is conducted to screen the molecular progress of polymerase complex of H9N2 virus in Egypt, sequencing of the three genes of the polymerase complex and molecular characterization of the obtained sequences were achieved. Our findings showed the presence of some point mutations in the three genes (PB2, PB1and PA) which had previously been linked to increased viral replication, virulence, pathogenicity of avian influenza viruses in mammals.

Key words: Avian influenza H9N2, PA, PB1 and PB2.

INTRODUCTION

The *Orthomyxoviridae* family includes avian influenza viruses (AIVs). In the United States, the avian influenza H9N2 virus was initially identified in turkeys in 1966 (Homme and Easterday, 1970). Saudi Arabia, Israel, Jordan, and Egypt are among the Middle Eastern nations where H9N2 virus has infected poultry (Golender et al., 2008).Due to their mammalian-like traits, H9N2 have crossed the species barrier resulting in mild to moderate infections(Nagy et al., 2017). Three hotspots for human AIV infections have reported a total of 27 laboratory-confirmed clinical illnesses in humans since March 2013, from them, four cases were in Egypt (Mostafa et al., 2018).

The entire genome of AIVs is made up of eight fragments, single-stranded, RNAs with negative sense that are tied by both the vRdRp and the nucleoprotein (NP) (Bouvier and Plese 2008). The vRdRp complex is comprised from polymerase basic 2(PB2), polymerase basic 1(PB1) and polymerase acidic (PA+PA-X)proteins

which are expressed from the first, the second and the third segments respectively. This complex is responsible for transcription and replication of viral RNA via cap-snatching mechanism, they transcribe virally encoded genes; then, they replicate full-length viral RNA to create first positive strand complementary RNA and progeny viral RNA. This replication mechanism affects the pathogenicity and virulence of AIV (Gabriel et al., 2005).

A key function in host shut-off mechanism is played by the protein PA-X, which is likewise generated from a portion of the PA gene segment. The shut-off process impairs the cell's expression machinery by causing its own RNA to degrade, which lowers host's mRNA levels and decreases host protein translation (Bercovich-Kinori et al., 2016).

In mammalian cells, the AIV polymerase complex performs irregularly, however some mutations can make it functional again. For example, multiple changes in the PA gene caused the 2009 pandemic H1N1 virus to initiate polymerase activity in mammalian cells (Lutz et al., 2020). Our study looked into molecular characteristics of the PB2, PB1 and PA genes of H9N2 influenza virus isolated from broiler chickens in Egypt in 2021 in order to determine point mutations (amino acid substitutions) related to mammalian preference and increased virulence.

METHODOLOGY

Sample collection:

A total of 25 broiler poultry flocks in Menoufia governorate were assessed for occurrence of avian fluvirus by collecting 50 oropharyngeal swabs during 2020–2021.

Viral RNA extraction:

After that, RNA was extracted from the samples using the QIAamp Viral RNA Mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany).

Realtime RT- PCR for detection of M gene:

The samples were applied to real time RT-PCR for detection of M gene (Spackman et al., 2003) by using QuantiTect probe RT-PCR catalogue No. 204443 that Affords precise concurrent quantification of viral RNA.

Viral isolation:

The M gene positive samples were individually injected into the allantoic cavity of specified pathogen-free embryonated hens' eggs aged 10 days. After the injection, 48 hours were spent incubating at 37 degrees Celsius, and then refrigerated for 4 hours. Then, allantoic fluid was gathered and underwent subtyping by RT-PCR.

Subtyping:

Viral RNA was extracted from the harvested allantoic fluids of the isolated samples by using of QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA., USA, Cat. No. 52904). RT-PCR was conducted for subtyping of HA and NA genes by using the following primers.

Table (1): Sequence of Primers used in the subtyping of avian influenza virus:

Target	Primer	Sequence (5′ to 3′)	Product size(bp)	Reference
H5	H5–155f	ACACATGCYCARGACATACT	545	Lee et al., 2001
	H5–699r	CTYTGRTTYAGTGTTGATGT		
H9	H9–151f	CTYCACACAGARCACAATGG	488	
	H9–638r	GTCACACTTGTTGTTGTRTC		
N2	N2-59F	TYTCTMTAACYATTGCRWCARTATG	278	Tsukamoto et al., 2009
	N2-336R	GARTT GTCYT TRGAR AAVGG		
N8	N8-93F	CATRTVGTBAGYATYAYARTAAC	137	

	N8-209R	ACAYTRGYATTGTRCCATTG		

Sequencing and Sequence Analysis:

One H9N2 isolate was subjected for sequencing of the polymerase complex genes (PB2, PB1 and PA) by using of Next Generation Sequencing (IonTorrent System). Raw data was analyzed by ion reporter software then obtained sequences were deposited in GenBank. Alignment of sequences was achieved by using Bio Edit software version 7.2.5.(Hall, 1999).It was possible to identify the genetic signature markers associated with virulence and host adaptability using the aligned amino acid sequence of each protein. The 3D protein structure of the Egyptian H9N2 virus was displayed by the SWISS-MODEL modeling online server (Arnold et al., 2006) and visualized by PyMOL 1.1 software (DeLano Scientific LLC).

RESULTS

Screening of samples and virus detection:

In this study, 11 out of 50 oropharyngeal swabs were positive for avian influenza virus by qRT-PCR (CT ranged from 15 to 21) as shown in figure 1.

Results of subtyping and sequencing:

The eleven samples were related to H9N2 subtype by conventional RT-PCR. The sequences identified in the present study can be found in the GenBank with the following accession IDs:
OP115742 (A/chicken/Egypt/Menoufia/2021(H9N2)) segment 1 polymerase PB2 (PB2) gene, complete cds, OP115741 (A/chicken/Egypt/Menoufia/2021(H9N2)) segment 2 polymerase PB1 (PB1) and PB1-F2 protein (PB1-F2) genes, complete cds andOP115743(A/chicken/Egypt/Menoufia/2021(H9N2)) segment 3 polymerase PA (PA)&PA-X protein (PA-X) genes, complete cds.

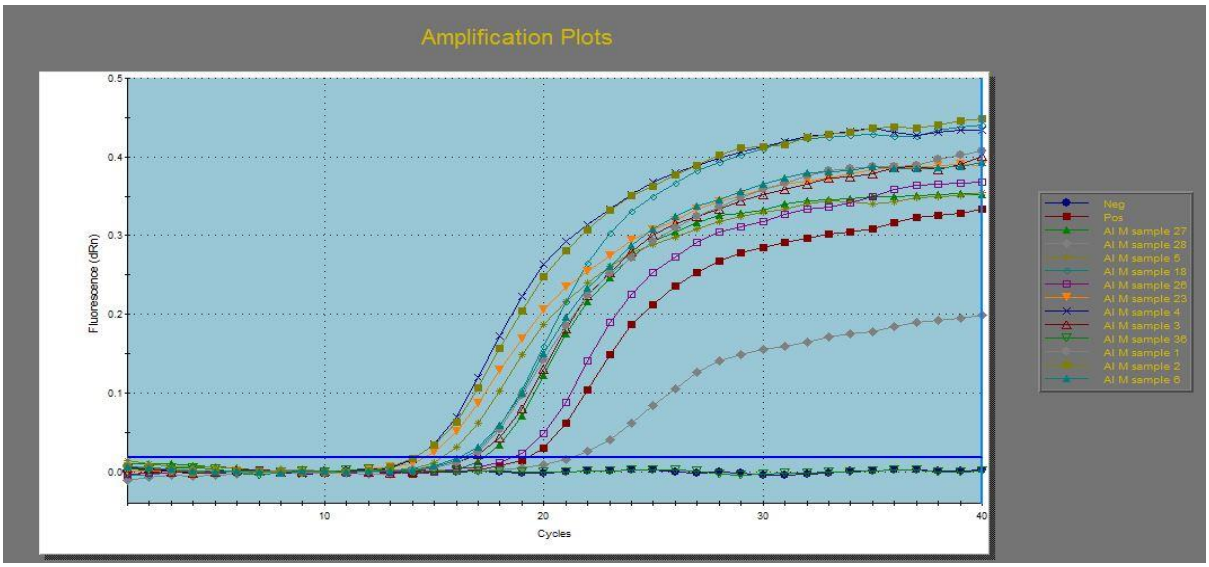

Fig. (1): Amplification curves of examined samples including negative and positive controls.

Molecular characterization:

In order to determine the molecular features of the polymerase complex genes of our Egyptian H9N2, deduced amino acids of PB2, PB1 and PA genes were aligned and compared with other Egyptian isolates. No deletions or insertions were observed in our Polymerase complex genes; however several point mutations were registered.

Amino acid sequences of the three genes of the polymerase complex were analyzed for the presence of molecular markers with documented roles in host tropism,

pathogenicity, and increased replication. The PB2 gene of our isolate has four amino acids substitutions; 147, 504V, K318R, and M64T as shown in the 3D protein structure in figure 2. The PB1 protein has 622G,R207K, H436Y and M677T amino acid substitution as shown in the 3D protein structure in figure 3 while the PA protein has six amino acids substitutions; N383D, N409S, 127V, 550L and 672L as shown in the 3D protein structure in figure 4.

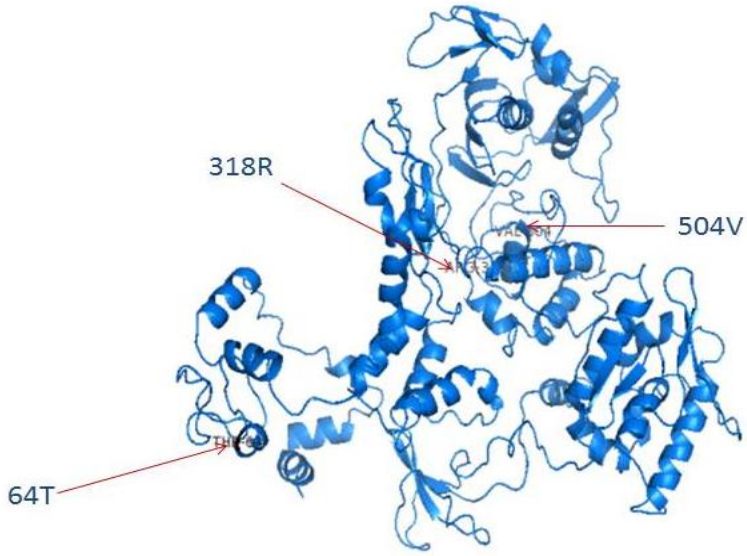

Fig. (2): 3D structure of PB2 protein, the amino acids at positions 64, 318 and 504 are threonine, arginine and valin respectively.

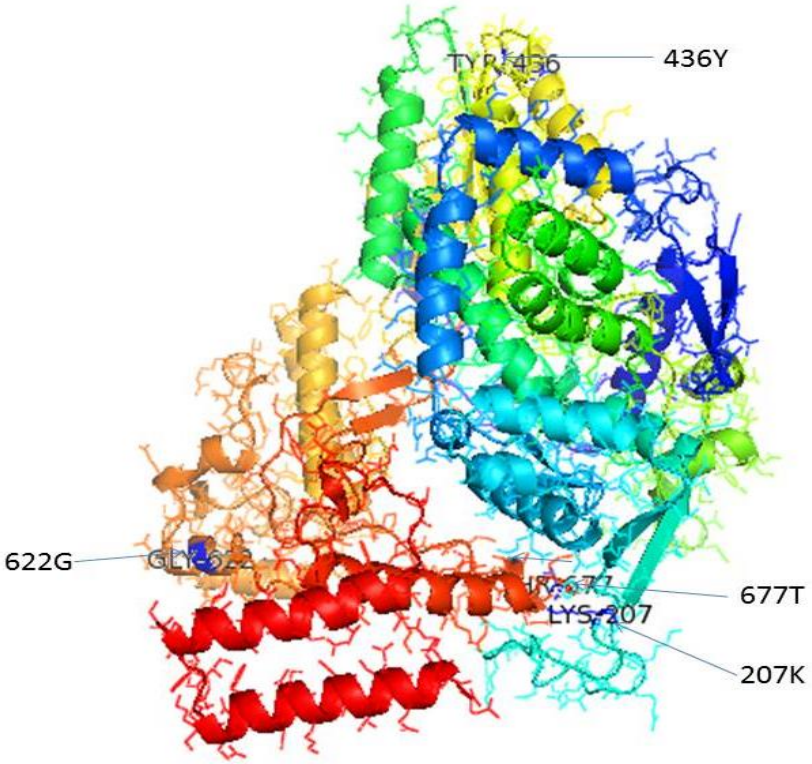

Fig. (3): 3D structure of PB1 protein, the amino acids at positions 622, 207, 436 and 677 are glycin, lysine, tyrosin and threonin respectively.

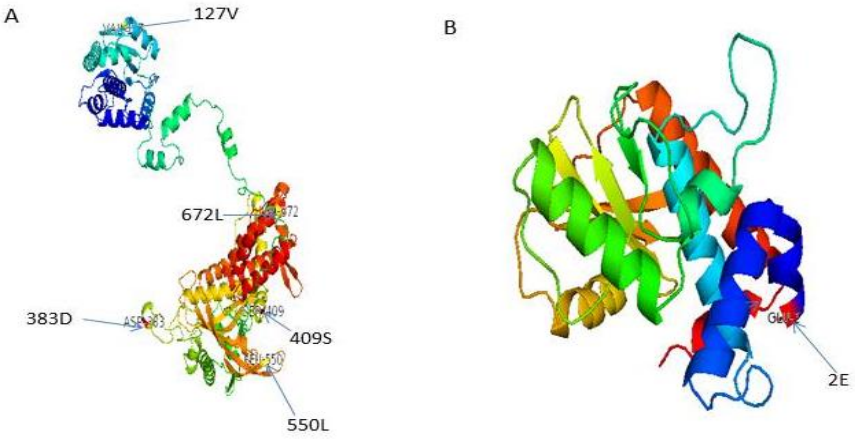

Fig. (4):(A) 3D structure of PA protein, the amino acids at positions 383, 409, 127, 550 and 672 are aspartic acid, serine, valin and leucin respectively. (B) 3D structure of PA-X protein, the amino acids at position 2 is glutamic acid.

DISCUSSION

It has been reported that the adjustment of novel emergent avian influenza viruses to human beings is a serious health risk which could cause the next pandemic outbreak. These avian influenza viruses

require adaptive alterations in specific viral genes to pass the species barrier and infect humans (Long et al., 2018). The HA and NA glycoproteins, which regulate viral entrance and emitting, are among these genes (Long et al., 2018). In addition, it has been discovered that the viral RNA-dependent RNA polymerase (vRdRp) is a key regulator of how well avian influenza viruses adapt to mammals (Lutz et al., 2020).

Different amino acid (aa) fingerprints, in particular, found in the PB2 protein are linked to the host range and effective proliferation of AIV in mammals. Many aa substitutions were detected in PB2 protein of our H9N2 isolate; M64T, K318R and K339T that could boost viral replication and make it more dangerous for mammals (Tombari et al., 2011). Moreover, I504V substitution was reported in PB2 protein of our H9N2 viruses and has been previously observed in Egyptian strains and is connected to increased polymerase activity (Rolling et al., 2009 and El Sayes et al., 2022).

The PB1 substitutions R207K, H436Y and M677T were reported to be associated with enhanced virulence and polymerase activity in mallards, ferrets and mice and may lead to high pathogenicity of avian influenza viruses (Hulse-Post et al., 2007) were identified in our Egyptian H9N2 isolate. In addition, D622G substitution was also detected in PB1 protein of our isolate which was associated with increased virulence and lethality of avian influenza viruses (Feng et al., 2016).

The PA protein of our H9N2 isolate has N383D and N409S substitutions that were experimentally confirmed to increase polymerase activity and viral replication (Song et al., 2015).127V, 550L and 672L residues were also detected in our isolate which are reported to increase virulence and pathogenicity of avian influenza viruses (Wanitchang et al., 2011).The third segment of avian influenza expresses also PA-X protein that functions a significant effect in the cellular shutoff activity. It has been shown that PA-X protein mutations render PA-X shut-off function entirely inactive (Khaperskyy et al., 2016). Three substitutions in the PA-X protein increase its role in the shut off activity (E2, P28L and S65L) (Dias et al., 2009). Our isolate has the E2 residue in PA-X protein.

In conclusion, our results suggest the ability of H9N2 to become highly pathogenic and emphasize the need for continuous surveillance and complete genomic characterization of currently circulating avian influenza viruses in Egypt to predict any upcoming epidemic strikes.

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