Virological and molecular diagnosis of avian influenza virus subtype h9 (h9)

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A B S T R A C T

Avian Influenza Virus (AIV) represents a dramatic pathogen among chicken flocks. The current study was carried out for isolation, identification and prevalence of LPAIVH9N2 from chickens and ducks in Damietta province. One hundred and twenty three (123) samples were collected randomly from broiler chickens in Damietta province in Egypt during 2014 – 2018.The samples were prepared, inoculated into ECE at age 9-11day without marked embryonic changes and even rapid slide HA. The samples were testing by real time RT-PCR that revealed 23 positive cases for AIV subtypes H5 and H9 with percentage of 8% and 10,5 % respectively .Analysis of the sequencing data of the (HA) revealed AIV H9N2 subtype in two samples had the mutation represented by (T3I, S5P, I9M, S11N, M58K and T143S) and (T3I, S5P, and S11N) in first and second sample respectively which is considered specific marker of influenza virus mutation. The Phylogenetic analysis of the HA gene showed that our two Egyptian isolates of H9N2 were resemble that isolated from Egypt in 2016 the G1-like lineage. Effective prevention is depend upon the insertion of such strains in any newly prepared vaccines and to construct a strategy for control the risk associated with spread of influenza caused by such virus.

Keywords: *AIV (H9N2), Isolation, HA, PCR, Sequencing, Mutation.*

1. INTRODUCTION

AIV belongs to the Family Orthomyxo-viridae and the Genus Influenza viruses A (Thomas and Noppenberger, 2007). The virus has a negative-sense, single-stranded, segmented RNA genome (eight segments) encoding at least 10 proteins: polymerase basic 1 (PB1), PB2, polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M), M2, nonstructural (NS1) and NS2. The HA and NA proteins are surface glycoproteins and important for virus infectivity. The HA protein is responsible for virus attachment to the host cell and the major target of the humoral immune responses. The NA protein plays a role in the increase and spread of progeny virions by removing sialic acid from glycoproteins.

AIVs infect both domestic poultry and wild birds; in addition, many reports have described their natural transmission to

humans and occasionally to other mammals (Kalthoff et al., 2010; Imai et al., 2013).

AIVs are classified, based on the presence of multiple basic amino acids at the cleavage site of their hemagglutinin (HA) protein and/or their virulence in chickens, as low pathogenic avian influenza(LPAI) or high pathogenic avian influenza (HPAI) viruses (Swayne,2013).

The lack of biosecurity measures for backyard birds in Egypt and their continuous contact with wild migratory birds make backyard birds much more vulnerable to AIV than those on commercial farms. The wild bird-backyard flock pathway was claimed to be the source of the first detection of H5N1 virus in Egypt in February 2006 (El-Zoghby et al., 2012).

A practical strategy for rapid, efficient and economical immunization of poultry in the field will be required for a better control of zoonotic avian influenza viruses for animal and public health. As a preventive strategy,

Table 1.Collection of samples:

Species	Number of samples
Chickens	108
Ducks	15
Total number of samples	123

2.2. Isolation of AIVH9 using ECE:

A total of 400 ECESPF (Kom Oshim - Fayoum) were used for inoculation and isolation of AIV via allantoic cavity of embryonic age 9-11day by 3egg/sample.The positive PCR samples were directed to virus isolation according to Abd El-Samie (2006), part I. Also heamagglutination test for AIV (H9N2) depending on Abd El-Samie, 2006, part II.

education of farmers for effective vaccination and enhancing biosecurity measures can also be beneficial in reducing the circulation of AIVs in the field (Kim, 2018).

Therefore, the current study was carried out for isolation, identification and prevalence of LPAIVH9N2 from chickens and ducks in Damietta province.

2. MATERIALS AND METHODS

2.1. Collection of samples:

One hundred and twenty three tracheal and cloacal swabbed samples were taken randomly from chickens and ducks from Damietta province in Egypt during 2014 - 2018. Swabs were placed in 1-2 ml isotonic phosphate buffered saline (PBS) PH 7.0- 7.4, chilled immediately on frozen gel packs and submitted to the laboratory as quickly as possible. Tracheal and cloacal swabs collected from broiler chickens and ducks flock as shown in table 1.

2.3. Molecular screening, detection of AIV using RT-PCR:

Viral RNA was extracted from both of field and ECE harvest homologously. samples were performed using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. Briefly 30μl of the sample suspension was incubated with 5.6μl of carrier RNA and 560μl of AVL buffer at room temperature for 10 min. After incubation, 560μl of 100% ethanol was added to the lysate. The mixture was then transferred to the silica column then centrifuged and washed following the manufacturer's instructions.

Viral Nucleic acid was eluted with 60μl of elution buffer provided within the kit. AI

H9 Primers were utilized in 25µl reaction containing 12.5µl of Quantitect probe RT-PCR master mix (Qiagen, Germany, GmbH), 0.5µl of each primer of 50 pmol concentrations, 0.125µl of the specific probe, 4.5µl of water, 0.25µl of the rt-enzyme and 6µl of RNA extract. The reaction was performed in a Stratagen MX3005P real time PCR machine (Stratagene, USA). Oligonucleitide primers are described as in table (2).

Table 2: RT- PCR Oligonucleitide primers used were supplied from Metabion (Germany).Three different sets of primers (forward and reverse) as subtype specific for H5, H7 and H9 of influenza type-A according to Chaharaein et al. 2009.

Primer	Target gene	Type	Sequence (5 - 3)
H5F	H5 gene	Forward	5' ACAAAGCTCTATCAAAACCCAAC 3'
H5R	H5 gene	Reverse	5' TACCCATACCAACCATCTACCAT 3'
H7F	H7 gene	Forward	5' CAGGCGGAATTGATAAGGAG 3'
H7R	H7 gene	Reverse	5' TGCCCCATTGAAACTGAAAG 3'
H9F	H9 gene	Forward	5' ATCGGCTGTTAATGGAATGTGTT 3'
H9R	H9 gene	Reverse	5' TGGGCGTCTTGAATAGGGTAA 3'

2.4. Molecular identification of AIV H9 isolates using Conventional RT-PCR:

By using QIAquick Gel Extraction Kit Protocol (Qiagen Inc. Valencia CA) for purification of PCR product from agarose gel. The PCR for amplification of partial hemagglutinin gene of H9 subtype was done by using specific primers. The electrophoresis of PCR products were done on ethidium bromide stained agarose gel and the amplified products of expected correct size were visualized by gel documentation system.

2.5. Molecular identification, sequencing, genotyping of AIV isolate:

Sequencing for two positive isolates was performed by purification of PCR products using QIAquick PCR Product extraction kit. (Qiagen, Valencia). The sequence reaction was done using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) and the sequence reaction was purified using Centrisep spin column (ABI, USA). DNA sequences were obtained using Applied Biosystems 3130 genetic analyzer (ABI, USA). A phylogenetic tree was created by the MegAlign module of Laser gene DNA Star. The sequencing method was done for HA gene of AI H9N2 virus.

3. RESULTS

3.1. Isolation of AIV using ECE: the samples which prepared were inoculated into ECE at age 9-11day without marked embryonic changes and even rapid slide HA.

3.2. Molecular screening, detection of AIV using RT-PCR: Testing of One hundred and twenty three samples by real time RT-PCR revealed 23 positive cases for subtype H5 and H9 of avian influenza virus with percentage of 8% and 10,5 % respectively, and threshold cycle ranged for positive results from 18 to 31.61 for H9 gene and 22.6 to 30.73 for H5 gene in chicken and 0%forH9 and 42.8for H5in ducks as shown in table 3and Fig 1 :

Table 3: Molecular screening for detection of AIV using RT-PCR in different species:

Species	No. of sample	Viral isolation	Molecular RT-PCR			
			C.TH5	C.TH9	+ve	
					H5	H9
Chickens	108	No clear embryonic changes even rapid slide HA	22.6-30.73	18-31.61	6	13
Ducks	15	No clear embryonic changes even rapid slide HA.	29.09-29.35	-	4	0
	123				10	13

CT= cut threshold
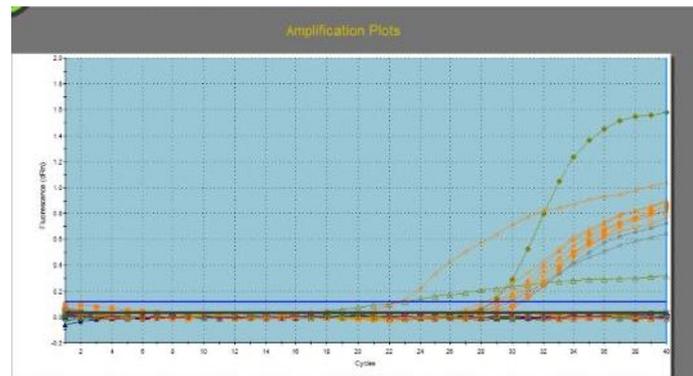
Fig (1): Molecular screening for detection of AIV using RT-PCR:
Molecular Identification of AIV isolates in samples collected from chickens and ducks in different locations in Damietta province using Real time RT-PCR showing positive c t value

3.4. Molecular identification, sequencing and genotyping of AIV isolate:

From 23 positive samples revealed by real time RT-PCR two selected isolates were positive for H9 subtype. The amplification of HA gene for the selected two isolates of study was conducted; the PCR products gave specific bands at 800 pb in weight for H9 isolates as shown in fig2.

The genetic analysis for 2 strains of AIV HA gene of the H9N2 type revealed that these viruses are low pathogenic as well as tracking G1 virus which is endemic and widespread in Egypt. In the phylogenic tree they are tracking G1-lineage of Asian viruses which spread at Middle East and they are nearly similar to other Egyptian viruses from 2011.

The phylogenetic relationship between the H9 gene of study isolate and those of selected H9N2 viruses isolated in Egypt and several other countries were analyzed. According to the phylogenetic tree of the nucleotide and amino acid sequences figures (3 &4), the two isolates of our study are related to the Egyptian viruses which are genetically related to G1 lineage of H9N2 avian influenza virus, the identity % between the two isolates and other Egyptian strains since 2011 ranged from 97-99% table (4).

Fig (2). Molecular identification, sequencing and genotyping of AIVH9 isolate:
The phylogenetic analysis of the H9 genomic sequence
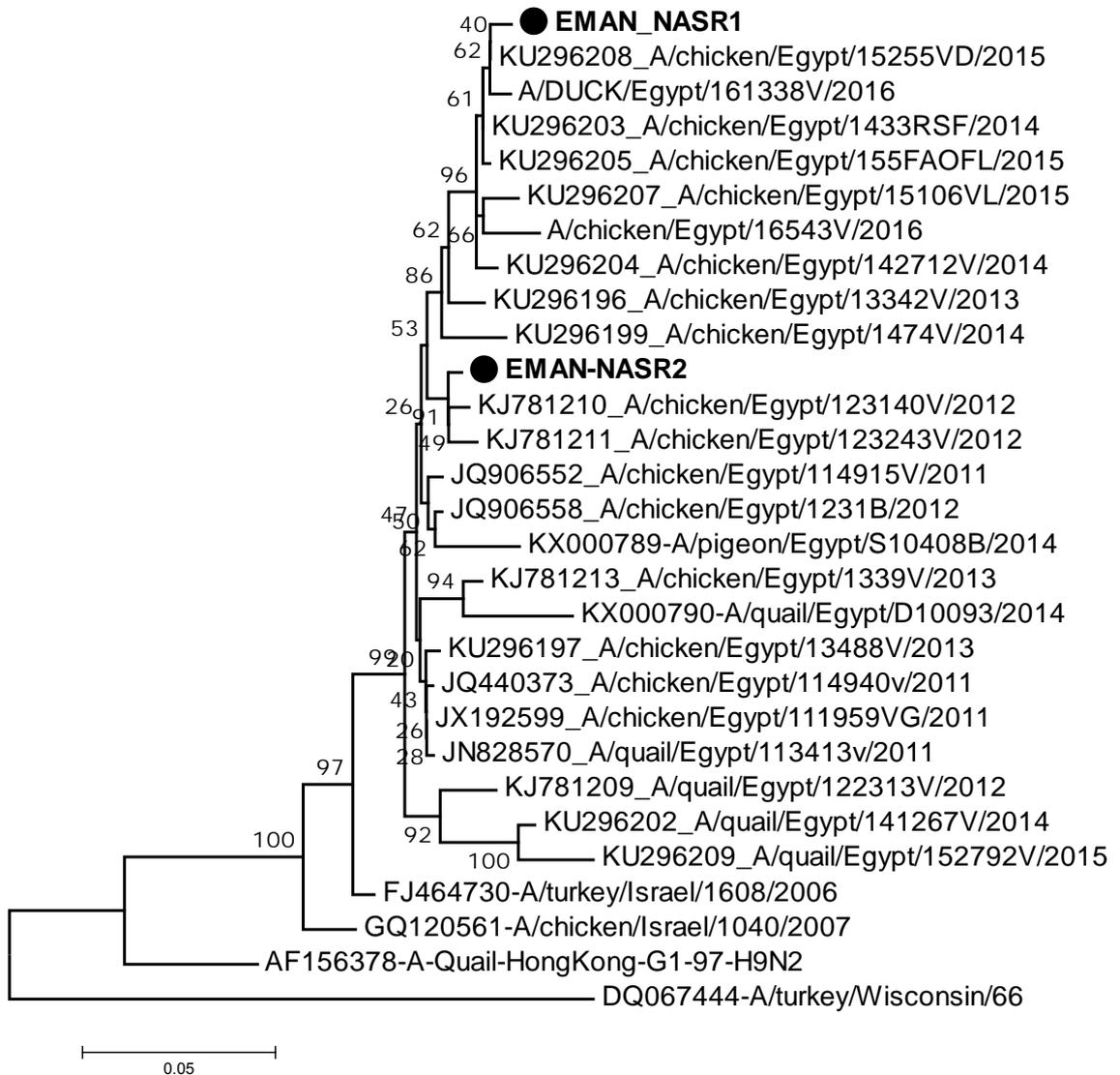
Fig. (3) Phylogenetic tree for nucleotide sequence for partial HA gene of H9N2, the samples of this study labeled with bold black circle.
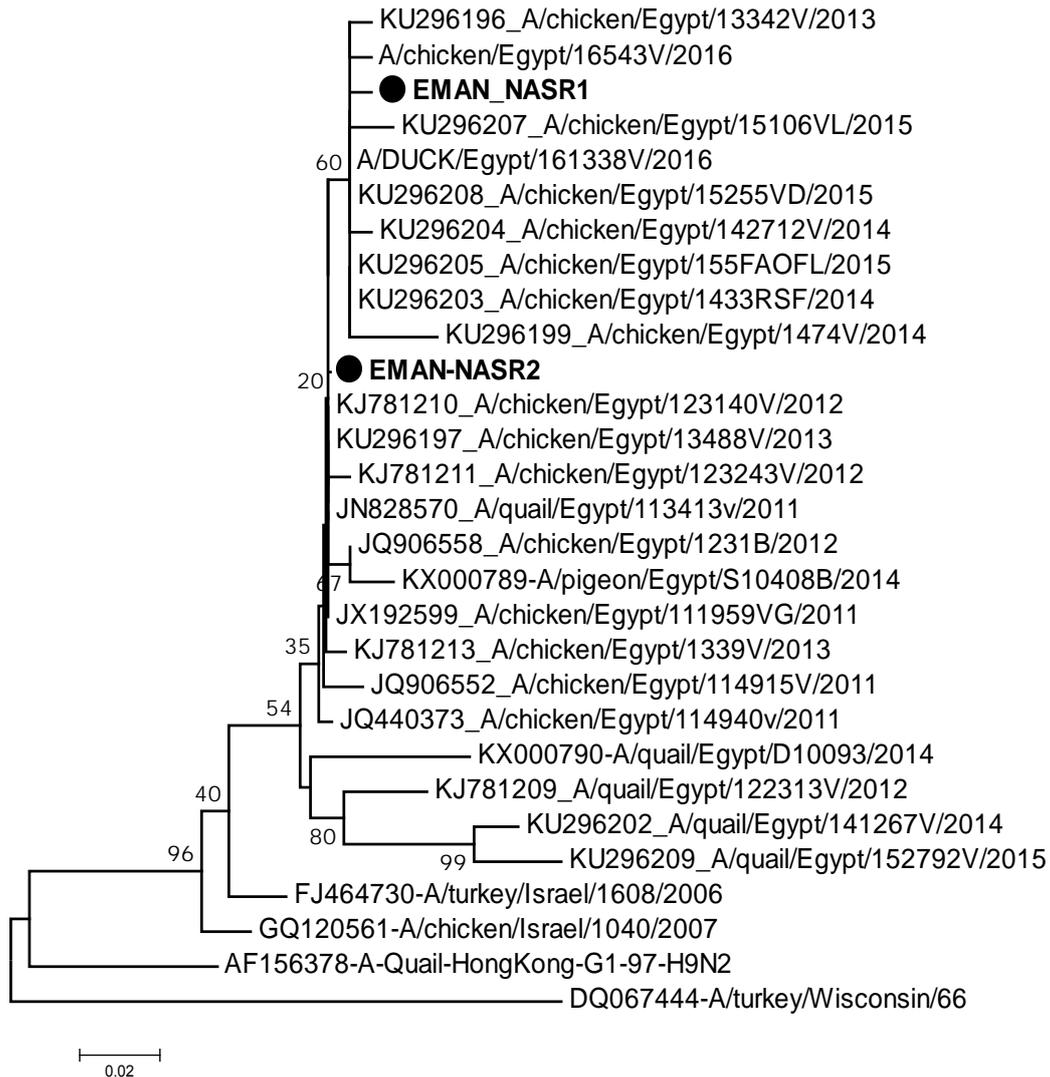
Fig. (4) Phylogenetic tree for amino acid sequence for partial HA gene of H9N2, the samples of this study labeled with bold black circle.

Table 4. The identity % among the different AIV isolates

	Seq- ID	1	2	3	4	5	6	7	8	9	10
1	EMAN NASR1		97%	97%	97%	96%	99%	98%	98%	87%	77%
2	EMAN-NASR2	99%		98%	98%	97%	98%	97%	96%	89%	78%
3	A/quail/Egypt/113413v/2011	99%	100%		99%	98%	98%	97%	96%	90%	78%
4	A/chicken/Egypt/1231B/2012	98%	99%	99%		98%	98%	97%	96%	89%	78%
5	A/chicken/Egypt/1339V/2013	98%	99%	99%	99%		97%	96%	95%	88%	78%
6	A/chicken/Egypt/1433RSF/2014	99%	99%	99%	99%	99%		99%	98%	88%	77%
7	A/chicken/Egypt/15106VL/2015	98%	98%	98%	98%	98%	99%		98%	87%	77%
8	A/chicken/Egypt/16543V/2016	99%	99%	99%	98%	98%	99%	98%		87%	77%
9	A-Quail-HongKong-G1-97	87%	88%	88%	88%	88%	88%	87%	87%		82%
10	A/turkey/Wisconsin/66	80%	80%	80%	80%	81%	80%	79%	80%	83%	

GAU Report 1
Code: V2041-2016
Analysis required: Sequence H9.
Number and type of sample: 1 clinical isolate.
Nucleotide sequence of chicken sample:

ATGGAAATAATACCACTGATGACTATGCTGTTATTAGTAACAACAAACAATGCAGACAAAATATGC
ATTGGCCATCAATCAACAAATTCTACAGAAACTGTGGACACACTAACGGAAACTAATGTTCCTGTG
ACACATGCCAAAGAATTACTCCACACAGAGCACAATGGAAAGCTGTGTGCAACAAATCTGGGAAA
CCCCCTCATCCTAGACACATGCACTATCGAAGGACTTATCTATGGTAACCCTTCTTGTGACATGTTG
TTGGGGGGAAGGGAATGGTCCTATATCGTTGAAAGACCATCAGCTGTGAATGGAACATGTTACCCT
GGGAATGTGGAAAACTTAGAGGAACTCAGAATACTTTTTAGTTCCTCTAGTTCATATCAAAGAATT
CAAATGTTCCCGGACACAATCTGGAACGTGAGTTACAGTGGAACAAGCAAATCATGTTCAGATTCA
TTCTACAGGAATATGAGATGGTTAACTCAAAAGAACGGGAATTATCCTGTTCAAGACGCCCAATAC
ACAAATACTCGGGGAAAGGACATTCTTTTCGTTTGGGGCATACATCATCCACCCACTGATACTGCA
CAGACGAATTTGTACACAAGAACCGACACAACAACAAGCATAACAACAGAAAATTTGGATAGGAC
CTTCAAACCATTGATAGGGCCAAGGCCCCTTGTCAATGGTCTGATTGGGAGAATTAATTATTATTG
GTCTGTACTAAAACCAGGCCAGACACTACGAGTAAGATCCAATGGGAATCTAATTGCCCCATGGTT
CGGACACGTACTCTCAGGAGAGAGCCATGGGAGAATTCTGAAAACTGATTTAAACAATGGCAATT
GTGTAGTGCAATGTCAGACTGAAAAAGGTGGCCTAAACAGTACATTACCTTTCCACAATATCAGTA
AATATGCATTTGGGGATTGTCCCAAATATATTGGAGTCAAAAGTCTCAAACTGGCAATCGGTCTGA
GAAATGTGCCTGCCAGGTCAAGTAGAGGGCTATTT

Comment

> The sample is positive by PCR and genetically characterized as Influenza A virus H9N2 similar to Influenza A virus (A/chicken/Egypt/1433RSF/2014(H9N2)) segment 4 hemagglutinin (HA) gene (Accession No. KU296203.1) , Influenza A virus (A/chicken/Egypt/F10533D/2015(H9N2)) segment 4 hemagglutinin (HA) gene (Accession No. KX000745.1) and Influenza A virus (A/Egypt/ ZU80/2016(H9N2)) segment 4 hemagglutinin (HA) gene (Accession No. KY910836.1).

Report 2

Code: F141-5   Date: 15-6-2018

Analysis required: Sequence H9.Analysis required: Sequence H9.
Number and type of sample: 1 clinical isolate.
Nucleotide sequence of sample of chicken:

ATGGAAATAATACCACTGATGACTATACTGCTATTAGTAACAACAAACAATGCAGATAAAATATGC
ATTGGCCATCAATCAACAAATTCCACAGAAACTGTGGACACACTAACGGAAACTAATGTTCCTGTG
ACACATGCCAAAGAATTGCTCCACACAGAGCACAATGGAATGCTGTGTGCAACAAATCTGGGTAA
TCCCCTCATCCTAGACACATGCACTATCGAAGGACTTATCTATGGCAACCCTTCTTGTGACATGTTG
TTGGGAGGAAGGGAATGGTCCTATATCGTTGAAAGACCATCAGCGGTAAATGGAACATGTTACCCT
GGGAATGTAGAAAACTTAGAGGAACTCAGAATACTTTTTAGTTCCTCTAGTTCCTATCAAAGAATT
CAAATGTTCCCAGACACAATCTGGAATGTGACTTACAGTGGAACAAGCAAATCATGTTCAGATTCA
TTCTACAGAAATATGAGATGGCTAACTCAAAAGAACGGGAATTATCCTGTTCAAGACGCCCAATAC
ACAAATACTCGGGGAAAGGACATTCTTTTCGTTTGGGGCATACATCATCCACCCACTGATACTGCA
CAGACGAATTTGTAcACAAGAACCGACACAACAACAAGCGTAACAACAGAAAATTTAGACAGGAC
CTTCAAACCATTGATAGGGCCAAGGCCCCTTGTCAATGGTCTGATTGGAAGAATTAATTATTATTG
GTCGGTACTAAAACCAGGCCAGACATTACGAGTAAGATCCAATGGGAATCTAATTGCTCCATGGTT
TGGACACGTTCTCTCAGGAGAGAGCCATGGGAGAATTCTGAAAACTGATTTAAACAGTGGTAATTG
TGTAGTGCAATGTCAGACTGAAAAAGGTGGCCTAAACAGTACATC

Comment

> The sample is positive by PCR and genetically characterized as Influenza a virus H9N2 similar to Influenza A virus (A/chicken/Egypt/D4692A/2012(H9N2)) segment 4 hemagglutinin (HA) gene (Accession No. JX912997.1), Influenza A virus (A/chicken/Egypt/123140V/2012(H9N2)) segment 4 hemagglutinin (HA) gene (Accession No. KJ781210.1) and Influenza A virus (A/chicken/Egypt/1256S/2012(H9N2) segment 4 hemagglutinin (HA) gene (Accession No. KJ781208.1).

4. DISCUSSION

AIV H9N2 is circulating worldwide in avian species, causing great economic

We collect One hundred and twenty three (123) samples randomly from broiler chickens in Damietta province in Egypt during 2014 – 2018.The samples were

prepared, inoculated into ECE at age 9-11day without marked embryonic changes and even rapid slide HA.

Conventional microbiological methods are sensitive and specific, it takes considerable time to reach a diagnosis; therefore, PCR-based detection and pathotyping assays have been developed to support/substitute for these traditional diagnostic tools (Wise et al., 2004). Real-time RTPCR can offer several advantages over standard RTPCR, such as speed and lower risk of cross contamination of clinical samples with previously amplified products In addition; the detection of the amplicon with a sequence-specific probe further confirms the nature of the target, reducing false positivity (Payungporn et al., 2006).

The viral RNA was extracted from both of field and ECE harvest homologously by QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. RT-PCR used for molecular characterizations of isolates using specific oligonucleotide primers for partial amplification of target genes of AI viruses (hemagglutinin gene (HA) gene).

Testing of the specimens by real time RT-PCR revealed 23 positive cases for subtype H5 and H9 of AIV with percentage of 8 % and 10,5 % respectively. And threshold cycle ranged for positive results from 18 to 31.61 for H9 gene and 22.6 to 30.73 for H5 gene.

Our results for AI virus detection by RT-PCR confirm the endemic picture of AI H5 with a percentage of 8% from total examined chickens and these observation agree with results of Aly et al. (2008) which recorded that the HPAI virus of subtype H5N1 was first recorded in Egypt in 2006 and since then, the disease has become endemic and still causes a significant threat to the poultry industry and humans in Egypt.

Detection of two subtypes of AI, HPAI H5N1 and LPAI-H9N2 in the present study and this conclusion discussed by Shortridge et al., 1998; Salzberg et al.; 2007 and Xu et al., 2007 who detected the presence of LPAI-H9N2 and the HPAI-H5N1 viruses have continuously co-circulated in domestic poultry in different areas.

The convential PCR for amplification of partial hemagglutinin gene of H9 subtype was done by using specific primers. The electrophoresis of PCR products were done on ethidium bromide stained agarose gel and the amplified products of expected correct size were visualized by gel documentation system.

. From 23 positive samples revealed by real time RT-PCR two selected isolates were positive for H9 subtype. The amplification of HA gene for the selected two isolates of this study was conducted; the PCR products gave specific bands at 800 pb in weight for H9 isolates.

On the other hand the Phylogenetic analysis of the HA gene showed that the 2 Egyptian isolates of H9N2 in our study were resemble that isolated from Egypt in 2011 the G1-ike lineage similar to the circulating viruses in the Middle East with very close phylogeny to the Israeli viruses and these results also resemble the results obtained by Arafa et al. (2012) when they recorded different strains of low pathogenic H9N2 in poultry sector.

The phylogenetic relationship between the H9 gene of this study isolate and those of selected H9N2 viruses isolated in Egypt and several other countries were analyzed.

According to the phylogenetic tree of the nucleotide and amino acid sequences, the 2 isolates of our study are related to the Egyptian viruses which are genetically related to G1 lineage of H9N2 avian influenza virus, the identity % between the 2 isolates and other Egyptian strains since 2011 ranged from 97-99%.

Phylogeographic analysis identified multiple introductions of influenza A (H9N2) virus into North Africa from the Middle East and Pakistan. The H9N2 subtype virus identified in Egypt seems to have originated from neighboring countries.

Analysis of the sequencing data showed that in the heamagglutination protein, the H9N2 subtype virus from Damietta had the mutation (T3I, S5P, I9M, S11N, M58K and T143S) and (T3I, S5P, and S11N) in first and second sample respectively which is considered specific marker of influenza virus mutation.

In this study, Influenza A virus H9N2 similar to Influenza A virus (A/chicken/Egypt/1433RSF/2014(H9N2)) was isolated from apparently healthy domestic chickens in the Damietta Province. Using the universal primer set, we determined the partial genomic sequence by reverse transcription PCR and direct sequencing for investigating the detailed genetic characteristics. The phylogenetic analyses of the H9 genomic sequence demonstrated that the HA gene shared the highest homology (above 99%) with that of a Egypt strain, A/quail/Egypt/113413v/2011 .

There are many alterations in the nucleotides and amino acids alignment among the Egyptian strains of our study and in comparison to the different lineages of H9N2 viruses. The molecular analysis focused on the pathogenic determinants of HA in comparison to A-Quail-HongKong-G1-97.

The Alignment report revealed presence of six Amino Acids differences in isolate eman nasr1 in compare with(A/Quail/Egypt/113413v-2011) which are: (T3I, S5P, I9M, S11N, M58K and T143S).The Alignment report revealed presence of three Amino Acids differences in isolate eman nasr2 in compare with (A/Quail/Egypt/113413v-2011) which are: (T3I, S5P, and S11N). Amino acids identity of H9N2 isolate of chickens obtained in this study in compare with (A/Quail/Egypt/113413v-2011) was 99%.

The current study described the genetic analysis of AIV H9N2 of HA gene that revealed these viruses are low pathogenic strains as they are tracking G1-lineage of Asian viruses which transmitted to Middle East since 2000. The analysis of isolated strains by different diagnostic and analytical techniques implies that the H5N1 and H9N2 infection exist in Egypt.

In conclusion, The Egyptian H9N2 viruses are continuously in genetic evolution, resulted in raising new variant viruses with antigenic variation. Thus, the Egyptian H9N2 viruses will be able to develop into more new variants adapted for transmission to other species. So, periodical and continuous surveillance is recommended to follow up the evolution of Egyptian viruses. Effective prevention is depend upon the insertion of such strains in any newly prepared vaccines and to construct a strategy for control of the risk associated with spread of influenza caused by such virus.

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