ElKersh *et al., J. of Virol. Sci.*, Vol. 6: 1- 11, 2019
ISSN: 2636-2937

Journal of Virological Sciences

RESEARCH

Molecular diagnosis of Avian Influenza Virus from different Avian species

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ABSTRACT

Background: The AIV is one of the most important poultry viral infections that represent one of the greatest concerns for public health. AIV classified within *Orthomyxoviridae* that is highly mutating virus. AIV was founded in Egypt since 2006. Control and prevention of AIV done via good managemental procedures, as well as strict quarantine measures and enhanced bio-security. Regular diagnosis and viral screening will be helpful in the eradication of the new strains to predict the newly coming waves of infection and to be able to develop the recommended vaccines.
Objective: Our study aimed for Molecular Diagnosis of *Avian influenza virus* (AIV) from Different Avian Species that would be useful to detect the current strains present in Egypt to use it in vaccination program.
Methods: In this study, we collected 122 sample representing 12 different area in Egypt and seven different species of poultry produced and traded in Egypt. These samples were isolated on SPF eggs, detected for AIV infection Via HA, RT-PCR and gene sequencing. We analyzed the sequence on gene bank to compare it with the other AIV strains
Results: We found 35 and 55 samples showed positive result for H5 and H9 respectively. After sequencing and comparing with the other AIV strains we found that the isolated strains showed close similarity with the AIV strains that were present in Egypt from 2006 to 2008 (subclade 2) and showed low similarity to the strains that were present in Egypt from 2008 to 2015.
Conclusion: AIV H5 strain that isolated from Chicken, Duck and pigeon, and AIV H9 strain that isolated from chicken show where mutated strains from previously AIV circulating in Egypt from 2006 to 2008. Therefore, we recommend incorporation of these such isolates in any newly prepared AIV vaccines and vaccination program.

Keywords: Avian Influenza; Virus; PCR; Sequencing

BACKGROUND

The AIV is one of the most important poultry viral infections that represent one of the greatest concerns for public health. AIV classified within *Orthomyxoviridae* that is pleomorphic with a size ranging from 80–120nm. (Swayne& Halverson 2008), enveloped and has a genome that consists of eight negative sense single stranded RNA segments that encode ten proteins. AIV is classified by the two surface antigens it carries in the envelope, heamagglutinin (HA) and neuraminidase (NA), there were 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) had been identified (Sun *et al.*, 2013) but recently H17N10 and H18N11 have been detected and maintained in aquatic birds and bats respectively (Adbel-Moneim *et al.*, 2009). There are different strains of AIV as it is divided in to low pathogenic (LPAIV) or high pathogenic (HPAIV). The geographical distribution of AIV covers worldwide and the HPAIV belongs to the H5N1 subtype are endemic in Asia and are spreading in Europe and Africa including Egypt. (Stallknecht *et al.*, 1990).

Control and prevention of AIV done via good managemental procedures, as well as strict quarantine measures and enhanced bio-security. After long period of using bio-security and

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vaccination, the AIV still able to make pandemics due to its ability to mutate, so regular diagnosis and viral screening will be helpful in the eradication of the new strains to predict the newly coming waves of infection and to be able to develop and improve the currently used vaccines. Our study aimed for Molecular Diagnosis of Avian Influenza Virus from Different Avian Species have been applied to detect the current strains that present in Egypt.

MATERIALS AND METHODS

Birds: a total number of 122 samples from different avian species (Table 2).
Universal transport medium such as M4, M4-RT, or VTM (multiple suppliers e.g. Remel, Lenexa). Vial of viral transport medium: Universal transport medium such as M4, M4-RT, or VTM. (Multiple suppliers: e.g. Remel, Lenexa, KS; Copan Diagnostics).SPF-ECE.Washed chicken red blood cells (RBCs).Antibiotic mixture.PCR Primer sets: primers and probes were manufactured in (Metabion, Germany) The sequence of primers & probe used in Real -Time PCR collected in Table (1).

Table 1: Oligonucleotide primers sets used for RT-PCR reaction for local AIV isolates:

M24	M gene	Forward	AGA TGA GTC TTC TAA CCG AGG TCG	(Spackman *et al*., 2002).
M25	M gene	Reverse	TGC AAA AAC ATC TTC AAG TCT CTG	
Probe M	M gene	Probe	6-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA	
H5LH1:	H5 gene	Forward	ACG TAT GAC TAC CCG CAG TAT TCA	
H5RH1:	H5 gene	Reverse	AGA CCA GCC ACC ATG ATT GC	
H5 Probe	H5 gene	Probe	6 -FAM-TCW ACA GTG GCG AGT TCC CTA GCA- TAMRA	
H9 -F0R	H9 gene	Forward	ATGGGGTTTGCTGCC	(Monne *et al.*, 2013)
H9 REV	H9 gene	Reverse	TTATATACAAATGTTGCAC(T)CTG	
H9-Probe	H9 gene	Probe	FAM-TTCTGGGCCATGTCCAATGG-TAMRA	

Electrophoresis reagents for RT-PCR products.Reagents used for Purification of DNA from PCR products: **(Fermentas):** Using Gene JET PCR purification kit # k0701 which contains: Gene Jet PCR purification Mini Spin Columns, binding buffer and wash buffer.
Elution buffer; sequencing and genetic analysis; dataset design: NCBI GenBank Influenza Virus Resource at NCBI according tohttp://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html (Bao *et al.*, 2008).Multiple Sequence Alignment: Multiple sequence alignment based on fast Fourier transforms (MAFFT). According to (Katoh *et al.*, 2002). Selecting the Best-Fit Evolutionary Model: jModel Test: phylogenetic model averaging (JAVA program that will run on Linux, Mac OS X, and Windows (Posada, 2008). Phylogenetic Reconstruction:Neighbor joining (NJ). PAUP*: (Swofford, 2001); Maximum likelihood (ML). GARLI: (Zwickl, 2006); bayesian inference (BI); MrBayes: (AlZanay *et al.*, 2011). Viewing and manipulating trees; Figtree. Collection and preparation of samples from different avian species.

Screening of the samples for AIV via Direct rapid slide HA.
Isolation, propagation and collection of AIV on ECE-SPF

Candling, inoculation, and collection of AIV from inoculated SPF- ECE then titration of propagated AIV and confirmation via HI according to (Abdelsamie, 2006).Molecular detection, identification, and diagnosis of AIV using RT-PCR then purification to the RT-PCR product, sequencing and gene analysis depending on the company protocol and using software and gene bank for gene analysis and Tree Visualization with Fig Tree according to (Madison,1997).

RESULTS

A total 122 samples collected from different avian species previously described in table (2) that the percentage of the birds' species to the total number of birds collected in (table3). The positivity of the results after isolation on SPF ECE and confirmation via HI were collected in table (4). The results of molecular identification via PCR were collected in table (5).

Table 2: Samples collected for detection of AIV

Area / Host	Chicken					Turkey					Duck					Quail					Pigeon					Total samples / Area
Sampling	N.S	O.S	H.C	I.O	F.F	N.S	O.S	H.C	I.O	F.F	N.S	O.S	H.C	I.O	F.F	N.S	O.S	H.C	I.O	F.F	N.S	O.S	H.C	I.O	F.F	
Cairo-Matrouh Sahrawi Road						1	1	2	1	1																6
Siwa Oasis			3								1	1	2	1	1											9
Dawli Sahili Road																		3								3
Nigila (Matrouh)			2																							2
ElHamam (Matrouh)	1	2	5	1	1																					10
ElSaloum (Matrouh)																							3	1	1	5
Biala (Kafr ELshiekh)																		2	6							8
Alexandria-Matrouh Road	2	2	12	2	2																					20
Baltim (Kafr Elshiekh)	2	2	12	2	2																					20
Sidi Brany (Matrouh)	3	1	12	3	1																					20
Wahat Road			14																							14
ElMathany (Matrouh)													5													5
Total Samples/ Number of Birds	89/ 70					6 / 3					11 / 8					11 / 11					5 / 4					122 / 96

N.S: Nasal swab, O.S.: Oropharyngeal swab, H.C.: Hole Caracas -of died bird, I.O.: Internal organs of life bird, F.F.: Fresh Fecal sample

Table 3: The percentage of birds' samples.

	Number of Samples and Bird type					
	Chicken	Tukey	Duck	Quail	Pigeon	Total
Total	89	6	11	11	5	122
%	72.95%	4.91%	9.02%	9.02%	4.1%	100%

Table 4: AIV isolation and identification Via HI**.**

	Number of Samples and Bird type					
	Chicken	Tukey	Duck	Quail	Pigeon	Total
Isolation on SFP ECE	65/ 89 73%Positive 24/89 27%Negative	6 /6 100%Negat-ive	5/ 11 45%Positive 6/11 55%Nega.	11/11 Negative	5/5 positive	75/ 122 61%Positive 47/122 39%Negative
HI Confirmation	60 positive	-	5 positive	-	5 positive	70 positive HA
Molecular Diagnosis PCR M Gene	40 positive	-	5 positive	-	5 positive	50 positive M gene
Molecular Diagnosis H5 & H9	30 H5 10 H9	-	5 H5		5 H5	40 H5 10 H9

Table 5: Molecular identification of AIV using PCR**.**

	Number of Samples and Bird type					
	Chicken	Tukey	Duck	Quail	Pigeon	Total
Molecular Diagnosis PCR M Gene	40 positive	-	5 positive	-	5 positive	50 positive M gene
Molecular Diagnosis H5 & H9	30 H5 16 H9	-	5 H5		5 H5	40 H5 16 H9

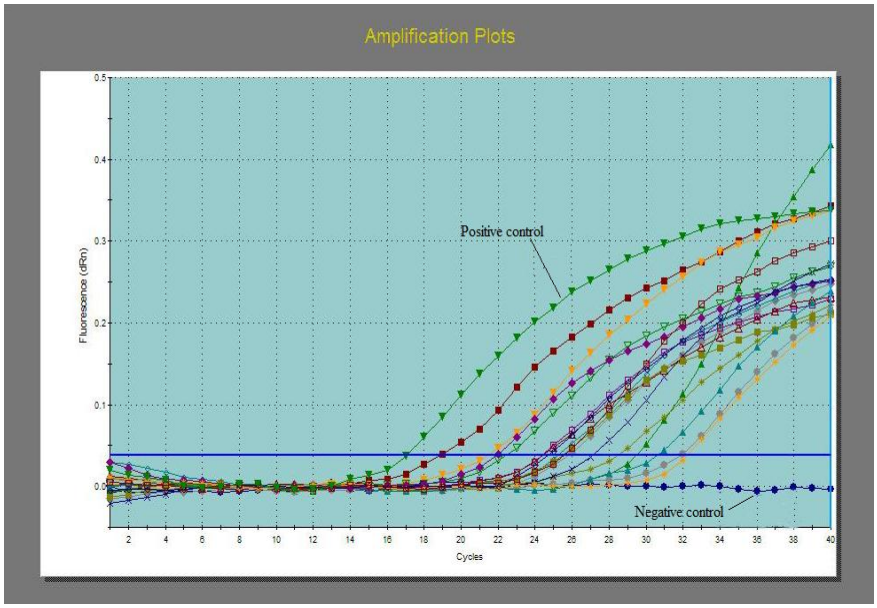


Fig. 1: Amplification curve for AIVH5 geneRRT-PCR where this fig. contain control positive, control negative and in between the control results; the curves of AIV H5 positive results for chicken, duck and pigeon.

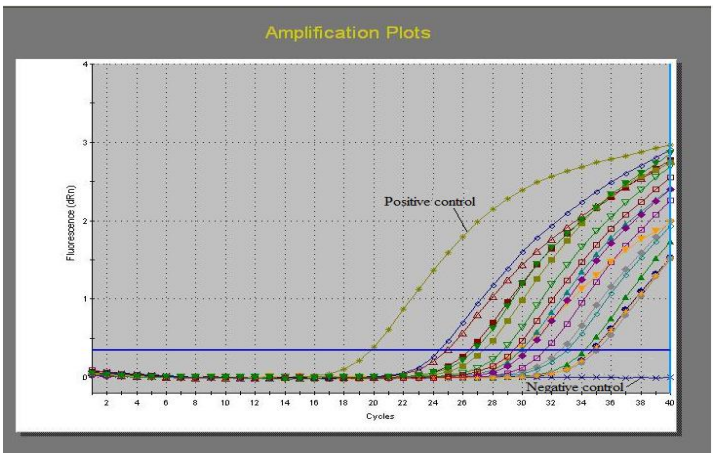


Fig. 2: Amplification curve for AIVH9 geneRRT-PCR where this fig. contain control positive, control negative and in between the control results the curves of AIV H9 positive results for chicken.

Molecular identification of AIV H5 isolated from chicken

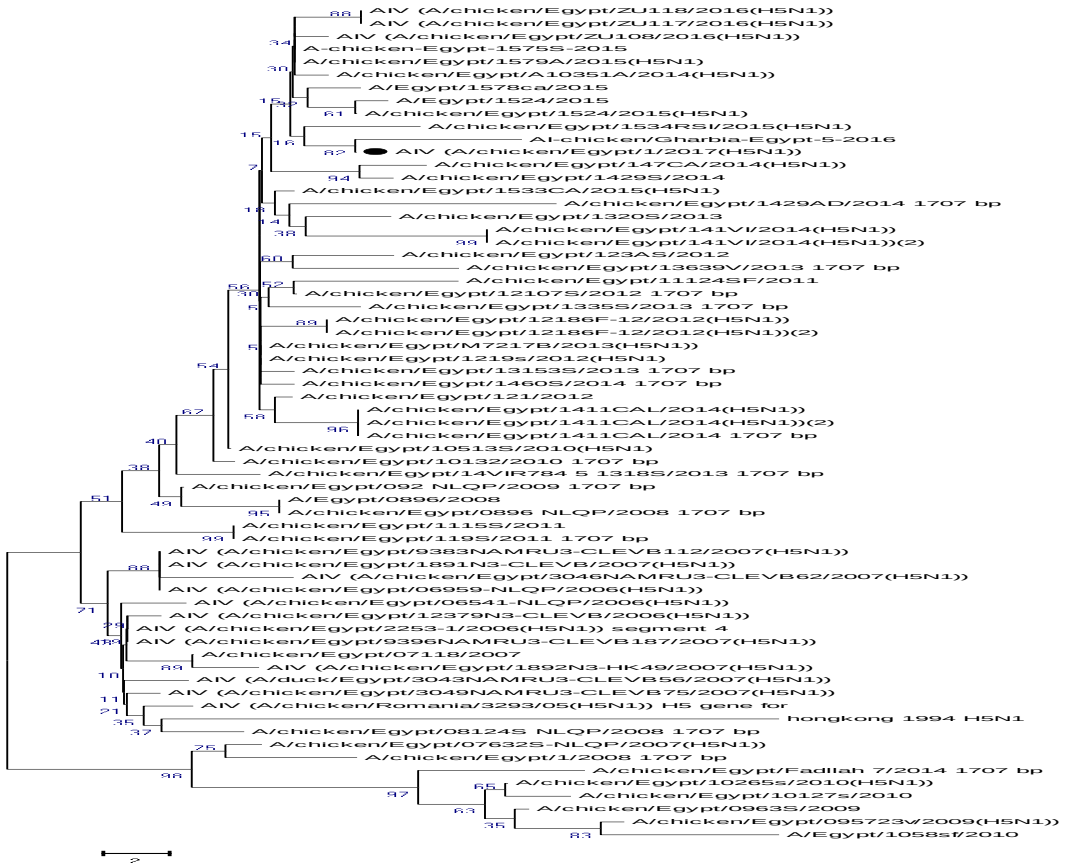


Fig. 3: Phylogenetic tree for the H5 AIV isolated from chicken and the related AIV strains based on the Nucleotides sequence.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1		97.6	97.1	91.3	95.6	97.5	96.4	96.7	96.4	94.9	97.1	95.9	87.9	96.9	1	AIV-(A-chicken-Egypt-06959-NLQP-2006
2	2.4		95.0	93.9	97.7	96.9	94.3	94.4	93.6	92.9	95.0	93.9	84.3	94.9	2	(A-chicken-Egypt-07632S-NLQP-2007(H5N1)
3	2.9	5.2		86.4	93.6	97.9	97.1	97.1	95.7	95.7	97.9	97.9	85.0	98.6	3	A-chicken-Egypt-0896-NLQP-2008
4	9.2	6.4	15.0		96.7	90.8	85.7	89.6	85.7	90.6	86.4	89.1	90.0	90.1	4	(A-chicken-Egypt-095723v-2009(H5N1))
5	4.6	2.3	6.7	3.4		95.4	92.9	92.9	92.1	91.9	93.6	92.4	83.6	93.4	5	(A-chicken-Egypt-10265s-2010(H5N1))
6	2.6	3.1	2.2	9.8	4.8		97.9	97.2	96.4	95.7	97.9	96.2	84.3	96.7	6	A-chicken-Egypt-119S-2011-1707-bp
7	3.7	6.0	2.9	15.9	7.5	2.2		98.6	96.4	95.7	98.6	98.6	84.3	97.9	7	(A-chicken-Egypt-12186F-12-2012(H5N1))
8	3.4	5.8	2.9	11.3	7.5	2.9	1.4		96.4	96.7	98.6	97.7	85.0	98.2	8	(A-chicken-Egypt-M7217B-2013(H5N1))
9	3.7	6.7	4.4	15.9	8.3	3.7	3.7	3.7		94.3	96.4	97.1	85.0	96.4	9	(A-chicken-Egypt-141VI-2014(H5N1))
10	5.3	7.5	4.4	10.1	8.6	4.5	4.4	3.4	6.0		95.7	96.7	87.9	97.2	10	A-chicken-Egypt-1534RSI-2015(H5N1)
11	2.9	5.2	2.2	15.0	6.7	2.2	1.4	1.4	3.7	4.4		98.6	85.0	98.6	11	AIV-(A-chicken-Egypt-ZU108-2016(H5N1))
12	4.2	6.4	2.2	11.9	8.1	3.9	1.4	2.3	2.9	3.4	1.4		85.0	99.0	12	AI-chicken-Gharbia-Egypt-5-2017
13	13.3	17.7	16.8	10.8	18.6	17.7	17.7	16.8	16.8	13.3	16.8	16.8		85.7	13	hongkong-1994-H5N1
14	3.1	5.3	1.4	10.7	6.9	3.4	2.2	1.8	3.7	2.9	1.4	1.0	15.9		14	AIV-(A-chicken-Egypt-1-2017(H5N1))
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		

Divergence

Fig. 4: AIV H5 identity to samples isolated from chicken compared with samples of most related isolates for amino acids identity percentage

Molecular identification of AIV H5 isolated from pigeon

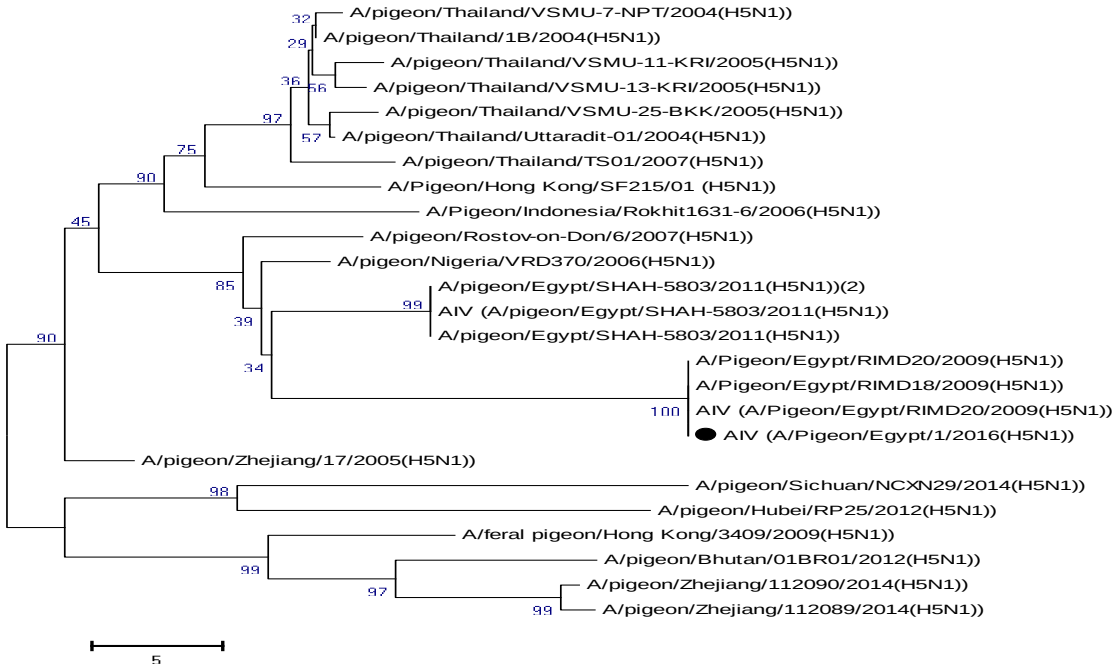


Fig. 5: Phylogenetic tree for the H5 AIV isolated from pigeon and the related AIV strains based on the Nucleotides sequence.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	
1		95.6	95.0	94.3	90.3	98.5	92.6	91.8	93.6	89.1	88.7	94.0	94.0	1
2	4.6		95.6	91.2	86.8	91.7	90.4	95.3	88.7	92.2	82.8	91.4	91.4	2
3	5.1	4.6		93.0	88.8	90.7	91.5	94.4	93.1	94.7	88.7	94.9	94.9	3
4	5.9	9.4	7.3		94.4	89.7	92.4	89.1	89.2	86.2	89.7	93.1	93.1	4
5	10.4	14.6	12.1	5.8		88.2	87.4	87.9	88.2	85.6	87.7	89.1	89.1	5
6	1.5	8.9	10.0	11.1	12.8		86.3	87.7	93.1	85.3	87.7	87.7	87.7	6
7	7.9	10.2	9.0	8.1	13.9	15.2		87.1	86.8	83.8	86.3	91.7	91.7	7
8	8.7	4.9	5.8	11.8	13.2	13.4	14.2		88.7	95.0	83.3	87.1	87.1	8
9	6.7	12.2	7.2	11.7	12.8	7.2	14.6	12.2		87.3	89.7	89.7	89.7	9
10	11.9	8.3	5.5	15.2	16.0	16.4	18.4	5.2	14.0		82.8	88.1	88.1	10
11	12.2	19.5	12.2	11.1	13.4	13.4	15.2	18.9	11.1	19.5		100.0	100.0	11
12	6.3	9.2	5.3	7.3	11.8	13.4	8.9	14.2	11.1	13.0	0.0		100.0	12
13	6.3	9.2	5.3	7.3	11.8	13.4	8.9	14.2	11.1	13.0	0.0	0.0		13
	1	2	3	4	5	6	7	8	9	10	11	12	13	

1 (A/pigeon/Thailand/Uttaradit 01/2004
2 (A/pigeon/nigeria/VRD/370/2006-H5N1)
3 A/FERAL PIGEON/HONG KONG/3409/2009
4 A/PIGEON/HONG KONG/SF215/01
5 A/PIGEON/THAILAND/TS01/2007
6 A/PIGEON/GHEJANG/112090/2007(H5N1))
7 A/PIGEON/HUBI/RP25/2012(H5N1))
8 A/PIGEON/ZHEJIANG/17/2005(H5N1))
9 A/PIGEON/ROSTOF ON- DON/6/2007(H5N1))
10 A/PIGEON/EGYPT/SHAH/5803/2011
11 A/PIGEON/EGYPT/RIMD20/2009(H5N1))
12 A/PIGEON/EGYPT/RTIMD18/2009(H5N1))
13 A/PIGEON/EGYPT/1/2016(H5N1))

Fig. 6: Identity of AIV H5 isolated from pigeon compared with samples of most related isolates for amino acids identity percentage

Molecular identification of AIV H5 isolated from Duck

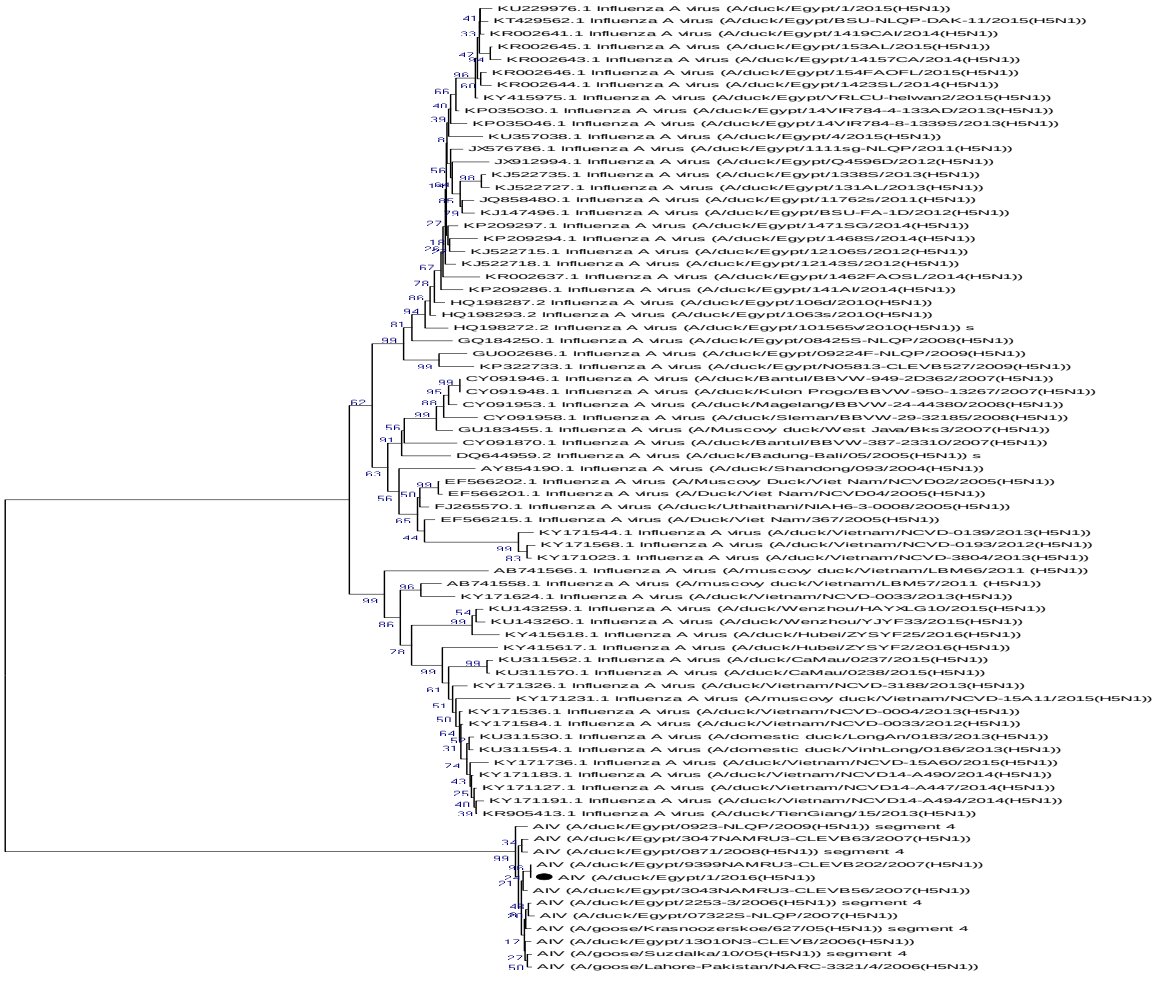


Fig. 7: Phylogenetic tree for the H5 AIV isolated from Duck,and the related AIV strains based on the Nucleotides sequence.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	
1		98.9	99.3	98.4	97.9	97.3	97.2	96.8	97.3	98.9	1
2	1.1		98.6	98.2	97.2	96.6	96.5	96.1	96.6	100.0	2
3	0.7	1.4		98.2	97.7	97.5	97.3	96.8	97.7	98.6	3
4	1.6	1.8	1.8		97.2	96.6	96.5	95.7	96.6	98.2	4
5	2.2	2.9	2.3	2.9		98.6	98.4	97.7	98.8	97.2	5
6	2.7	3.5	2.5	3.5	1.4		98.8	98.4	99.1	96.6	6
7	2.9	3.6	2.7	3.6	1.6	1.3		98.0	98.6	96.5	7
8	3.3	4.0	3.3	4.4	2.3	1.6	2.0		98.2	96.1	8
9	2.7	3.5	2.3	3.5	1.3	0.9	1.4	1.8		96.6	9
10	1.1	0.0	1.4	1.8	2.9	3.5	3.6	4.0	3.5		10
	1	2	3	4	5	6	7	8	9	10	

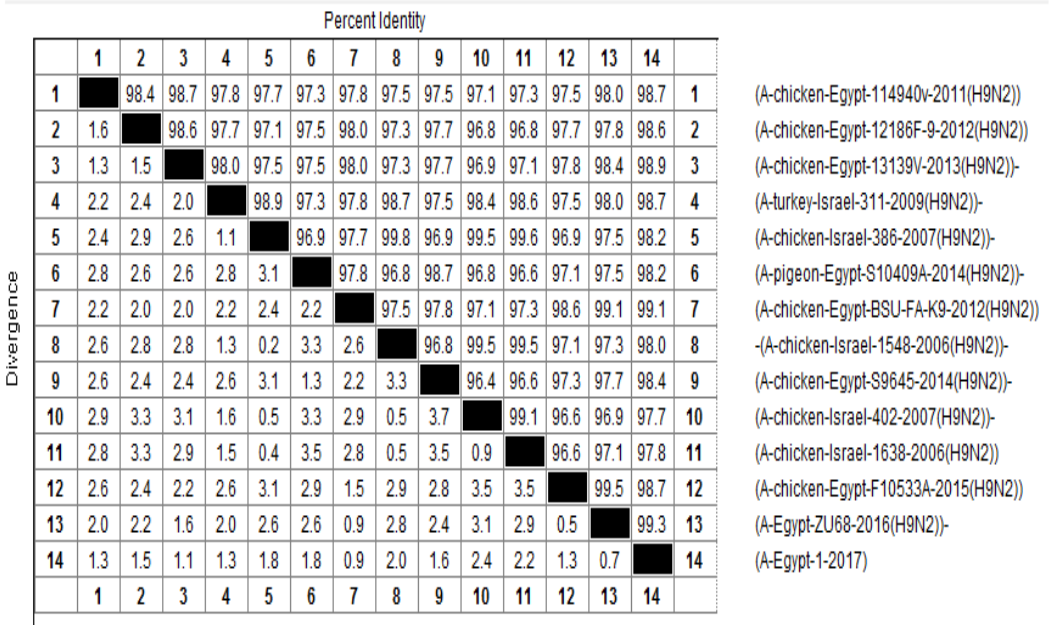
Divergence

1 AIV-(A-duck-Egypt-2253-3-2006(H5N1))
2 AIV-(A-duck-Egypt-9399NAMRU3-CLEVB202-2
3 AIV-(A-duck-Egypt-0871-2008(H5N1))
4 AIV-(A-duck-Egypt-0923-NLQP-2009(H5N1))
5 AIV-(A-duck-Egypt-1063s-2010(H5N1))
6 AIV-(A-duck-Egypt-1111sg-NLQP-2011(H5N1
7 AIV-(A-duck-Egypt-12106S-2012(H5N1))
8 AIV-(A-duck-Egypt-1338S-2013(H5N1))
9 AIV-(A-duck-Egypt-141AI-2014(H5N1))
10 AIV-(A-duck-Egypt-1-2016(H5N1))

Fig. 8: AIV H5 identity to samples isolated from Duck compared with samples of most related isolates for amino acids identity percentage

Molecular identification of AIV H9 isolated from Chicken

Fig. 9: Phylogenetic tree for the H9 AIV isolate and the related AIV strains based on the Nucleotides sequence.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1		98.4	98.7	97.8	97.7	97.3	97.8	97.5	97.5	97.1	97.3	97.5	98.0	98.7	1
2	1.6		98.6	97.7	97.1	97.5	98.0	97.3	97.7	96.8	96.8	97.7	97.8	98.6	2
3	1.3	1.5		98.0	97.5	97.5	98.0	97.3	97.7	96.9	97.1	97.8	98.4	98.9	3
4	2.2	2.4	2.0		98.9	97.3	97.8	98.7	97.5	98.4	98.6	97.5	98.0	98.7	4
5	2.4	2.9	2.6	1.1		96.9	97.7	99.8	96.9	99.5	99.6	96.9	97.5	98.2	5
6	2.8	2.6	2.6	2.8	3.1		97.8	96.8	98.7	96.8	96.6	97.1	97.5	98.2	6
7	2.2	2.0	2.0	2.2	2.4	2.2		97.5	97.8	97.1	97.3	98.6	99.1	99.1	7
8	2.6	2.8	2.8	1.3	0.2	3.3	2.6		96.8	99.5	99.5	97.1	97.3	98.0	8
9	2.6	2.4	2.4	2.6	3.1	1.3	2.2	3.3		96.4	96.6	97.3	97.7	98.4	9
10	2.9	3.3	3.1	1.6	0.5	3.3	2.9	0.5	3.7		99.1	96.6	96.9	97.7	10
11	2.8	3.3	2.9	1.5	0.4	3.5	2.8	0.5	3.5	0.9		96.6	97.1	97.8	11
12	2.6	2.4	2.2	2.6	3.1	2.9	1.5	2.9	2.8	3.5	3.5		99.5	98.7	12
13	2.0	2.2	1.6	2.0	2.6	2.6	0.9	2.8	2.4	3.1	2.9	0.5		99.3	13
14	1.3	1.5	1.1	1.3	1.8	1.8	0.9	2.0	1.6	2.4	2.2	1.3	0.7		14
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	

Fig. 10: AIV H9 isolated from chicken identity compared with samples of most related isolates for amino acids identity percentage.

DISCUSSION

Extensive surveillance and genetic studies revealed that H5N1 viruses had become endemic in poultry in many countries since 2003 ***(*Li *et al.*, 2004)*** and from 2007 this was true for Egypt. Although the promoters of endemicity in Egypt had not clarified yet. Silently, mixed species backyard holdings suspected to play essential role in the region. These domestic bird holdings are difficult to control. Culturally and socially coined poultry rearing as well as the predominance of live bird trading habits and economically related hesitant responses to public programs trying to raise awareness towards the potential dangers are at the basis of the endemic status of HPAIV H5N1.

A total number of 122 samples collected from 5 Egyptian areas included (Cairo Matrouh Sahrawy Road, Cairo Wahat Road, Dawli Sahili Road, Siwa & Matrouh Provence and Kafr Elshiekh Provence) were examined.

We identified the AIV isolates via isolation on SPF-ECE, screening of AIV isolates with rapid HA, then HI, and molecular detection of H5& H9 using PCR in the recommended cascade using different primers and probes. then sequencing of the PCR products**.**

Phylogeny of H5 gene clustered the Egyptian H5N1 viruses with those isolated from chickens in Egypt within the subclade 2.2.1. The later express relatedness to others, suggesting a single source of infection usually from backyards with further spread that seems to be related to trade of live birds which is most probably the source of infection.

There 3 isolates representing different bird species and different areas in Egypt were fully sequenced and were compared with other selected (H5N1) and (H9N2) strains from Egypt and different countries. The data revealed that all Egyptian strains were very closely related and belonging to clade 2.2 of the H5N1 virus of Eurasian origin, the same one circulating in the Middle East region and introduced into Africa at the beginning of 2006 (Aly *et al.*, 2008).

The isolated AIV from pigeon is similar to the virus strain that isolated in 2009 and this result was in close similarity with (El-Zahed *et al.,* 2015) that said the HA gene sequence of 6 selected isolates from 17 positive real time RT-PCR was evaluated. The results revealed that 5 isolates were H5 subtype and one isolate was H9 subtype. The phylogenetic analysis of H5 viruses indicates their relatedness to classical strain, while the H9 virus was related to G1-like lineage. The sequence analysis of H5 nucleotides and amino acids revealed that all isolates are closely related to the first avian influenza 2006 isolate in Egypt, while H9 isolate showed slightly different from the first H9N2 avian influenza isolated.

The vaccination with AIV H5 and H9 will decrease the speared of infection and appearance of apparently healthy birds (Naguib *et al.,* 2017). Vaccination considered as one of the major options for controlling avian influenza (Monto, 2003). Due to mutations in the influenza virus genetic material creating antigenic drift of pathogenic viral proteins resulting in emergence of new influenza virus strains. Therefore, the vaccines available for prevention of influenza offer no protection against influenza pandemics caused by new virus strains (Banerjee and Kaul, 2010). In addition, it was documented that the extensive vaccination of poultry had been resulted in the emergence of vaccine escape mutants (Lee *et al.,* 2009).

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RECEIVED: Jan, 2019; **ACCEPTED:** March 2019; **PUBLISHED:** July 2019

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Cite this article as:
ElKersh *et al.*, (2019): Molecular diagnosis of Avian Influenza Virus from different Avian species. *Journal of Virological Sciences*, Vol. 6: 1- 11.