Mansoura Veterinary Medical Journal

STUDIES ON H9 AVIAN INFLUENZA IN DAKAHLIA GOVERNORATE

*Asmaa A. Alzayat, **Abdelfattah H. Eladl,***Hanan A. Fahmy, ****Hany F. Ellakany

*Department of Poultry Diseases, Animal Health Research Institute, Mansoura branch, Egypt.
**Department of Poultry Diseases, Faculty of Veterinary Medicine, MansouraUniversity,Egypt.
***Department of Biotechnology, Animal Health Research Institute, Dokki, Egypt.
****Department of Poultry Diseases, Faculty of Veterinary Medicine, Damanhour University, Egypt.

ABSTRACT

Prevalence of H9 Avian Influenza Virus (AIV-H9) in Dakahlia governorate during 2012 - 2015 was undertaken to update the epidemiology of avian influenza virus infections among poultry flocks in Egypt. A total of 50 chicken flocks were examined (28 broiler flock and 22 layer flock). Results revealed that 18 samples were HA positive while 7 out of 18 samples were positive by chromatographic strip test for common antigen of AIV. Two chicken flocks were found positive AIV-H9 by RT-PCR and qrt-PCR. The virus was successfully isolated and propagated in SPF eggs. The result was screened by HA and confirmed by chromatographic strip test, RT-PCR and qrt-PCR. The PCR tests confirmed that the 2 isolates (12B and 19L) were free from contamination with H5 and ND. Both isolates (12B and 19L) were titrated giving EID_{50} $10^{8.5}$ and 10^{8}. Pathogenicity index (PI) of the isolates (12B and 19L) was assessed experimentally 0.8 and 1.1. Experimentally, the results also revealed faster mortality occurred with both H9 infection and IB live strain vaccination. In conclusion, our results confirmed the continuous circulation of the AIV- H9 in chicken flocks in Dakahlia governorate which exaggerate the respiratory problems in poultry farms.

Keywords: *AIV- H9, prevalence, RT-PCR, Pathogenicity index, Dakahlia.*

INTRODUCTION

Avian influenza viruses (AIV) strains including H9 strain belong to *Orthomyxoviridae* family type A **(Tong *et al.*, 2013)** which are further classified according to pathogenicity into highly pathogenic avian influenza (HPAI) such as H5, H7 which cause high mortality in poultry reaching up to 100 % and low pathogenic avian influenza (LPAI) such as H9 that show little signs and barely causing mortality **(Halvorson, 2002)**.

The cleavage is a must for the virus to be infective, H9 cleaved locally by trypsine like enzymes in the body of the bird in localized area such as trachea, lung and intestine so causing mild disease with low mortality not exceed 10% **(Guo *et al.*, 2000)**. While in case of bacterial infection cleavage occurs by bacterial protease which spread the infection to further organs causing more sever condition and mortality that may reach up to 80 %**(Kishida *et al.*, 2004).** Also A trypsin-like serine protease domain is encoded by infectious bronchitis IBV which exacerbate disease condition by both infection and vaccination with live strains of IB **(NG and Liu, 2000 and Karimi-Madab *et al.*, 2010**).

Amino acid sequence PARSSR/G at the HA cleavage site, a hallmark of low-pathogenic AIV (**Zhao *et al.*, 2013)**. Recent H9N2 isolates have HA cleavage site with dibasic (R-S-S-R)

or tribasic (R-S-K/R-R) motifs but suitable mutations could change the heamagglutinin cleavage site to be recognized by furin, resulting in highly pathogenic avian influenza **(Aamir *et al.*, 2007 and Xu *et al.*, 2007)**.

It has been reported that H9 virus has been crossed the species barrier to infect human directly without prior adaptation causing mild disease condition. The risk in some H9 virus is that it can carry one or more gene from highly pathogenic H5 **(Guo *et al.*, 2000)**, receptor-binding preference for a2, 6-linked sialic acid **(Sorrell *et al.*, 2009)** and preferably infect non ciliated cells and replicated more efficiently in human airway epithelial cell cultures which increasing the infection severity in humans (**Wan and Perez , 2007)** which with a little mutation may cause new pandemic of influenza in humans with human-to-human transmission **(Butt *et al.*, 2005)**.

In this study, we planned to detect AIV-H9 in Dakahlia governorate, isolate, screen the isolate with HA and confirm the isolates with rapid chromatographic strip test ,qrt-PCR and RT-PCR, measuring the pathogenicity of the isolates experimentally and study the effect of H9 infection with vaccination against ND & IB.

MATERIAL AND METHODS

Clinical samples: Fifty chicken flocks (28 broilers and 22 layers) of different breeds, ages and localities (2012-2015) were examined in Dakahlia governorate **(Table 1&2).** Samples were collected from broiler flocks that showed respiratory distress and variable mortality rates and from layer farms that suffered from respiratory manifestations and drop in egg production. Samples (trachea and lung) were collected from freshly dead birds then frozen at -20°C till used. Tracheal and cloacal swab samples collected from live or freshly dead birds and frozen at -20°C till used. Samples were prepared according to **OIE (2014).**

Embryonated chicken eggs: Prepared samples inoculated in allantoic cavity of SPF eggs (4 eggs per sample) in National Laboratory for quality control of poultry production (NLQP) according to **(OIE 2014)** at Dokki, Giza, Egypt**.** Collected allantoic fluid was stored at -20°C or -80°C for further examinations.

Plate hemagglutination test: The test was done as described by **Beard (1989).**

Chromatographic strip test: The Positive hemagglutinating samples were screened by rapid chromatographic strip test *(ANIGEN® animal genetics, Inc. Korea)* which is rapid AIV common antigen test kits. The procedure was done according to the manual supplied by the manufacture company **(Cui and Tong, 2008)**.

Quantitative rt-PCR (qrt-PCR): Suspected allantoic fluids were kept in ice and transported to National Laboratory for quality control of poultry production (NLQP)**.**Viral RNA was extracted from allantoic fluid by using a QIAamp Viral RNA Mini Kit (QIAGEN) catalogue No. 52904 according to the manufacturer's directions. Using QuantiTect probe RT-PCR catalogue No. 204443 for preparation of PCR Mastermix which provides accurate real-time quantification of RNA targets. Preparation of PCR Master Mix according to QuantiTect probe RT-PCR.Using Real time PCR machine (Stratagene MX3005P), the thermal profile has been adopted so as to detect the distinct subtypes at the same time and within the same run. The following thermo-cycling protocol was designed for all primer/probe **(Table 3)** sets: 20 min at 50°C and 15 min at 95°C, followed by 40 cycles at 94°C for 45 s and 54°C for 45 s.

Titration of the isolated samples:

Tenfold serial dilutions of the 2 isolates has been made and each dilution inoculated in the allantoic cavity of 5 SPF eggs 9-10 day old, incubated at 37°C for 2days, candled daily and dead ECE chilled overnight. Allantoic fluid collected and plate HA test was done for each egg separately and the result recorded **(Villegas and Purchase, 1989).** EID_{50} measured according to **Reed and Muench formula (1938).**

Experimental design:

This experiment was planned to study the effect caused by H9 infection ($EID_{50}=10^{6.5}$) **(Aslam *et al.*, 2015)** after the vaccination with MA5 (*Intervet*®,lot No. A183b1J01, $>=10^3$ EID_{50}) & Clone 30 (*Bioveta*®, lot No. 315721A, $>=10^6$ EID_{50}) against both IB & ND respectively. Six groups of broiler chicken (21 days), 20 birds in each group, were observed for mortality per day as shown in **table (8)**. Postmortem lesions were also observed in dead birds. Serum samples collected pre & post-challenge kept at -20°C for further examination. Organs (lung, trachea) and swaps (tracheal & cloacal) had been collected kept in -20°C &-80°C for further examinations**.**

Assessment of Pathogenicity:

According to **OIE (2014)** pathogenicity of titrated isolates has been assessed by intravenous route (I/V). Fresh infective allantoic fluid with a HA titer >1/16 is diluted 1/10 in sterile isotonic saline. 0.1 ml of the diluted virus is injected intravenously into 6-week-old male Hy-line chickens, birds were examined at 24 hour intervals for 10 days, each bird was scored 0 if normal, 1 if sick, 2 if severely sick and 3 if dead and pathogenicity index estimated . While for oculonasal route according to **Motamed *et al.*, (2013)** diluted virus with different concentrations (1/10, 1/100, 1/500, 1/1000) were dropped intranasal & intraocular, observed at 24hours interval for 10 days.

In our research, the pathogenicity of isolate12B with EID_{50} $=10^{8.5}$was assessed in male Hy-line birds through I/V route and oculonasal route, the later with different concentrations using 10 male Hy-line breed (42 day age) for each. Also the pathogenicity was assessed for isolate 19L through oculonasal route with concentration of 1/1000 (EID_{50} $=10^5$) in 10 male Hy-line breed **(Table 7)**. Different concentrations of virus were prepared by adding calculated amounts of saline on exact amount of allantoic fluid containing virus for example; 1/10 was prepared by adding 100µl virus to 900 µl saline.

Haemagglutination inhibition test: The test was done according to **Beard (1989)**.

Statistical analysis: All statistical analysis analyzed by the student t-test after **Steel and Torrie (1980)** to determine the significance of differences between groups.

RESULTS

Clinical findings of naturally infected birds: The examined broiler flocks showed general signs of illness in form of depression, anorexia, reduced water and feed intake, ruffled feathers, coughing, tracheal rales , sneezing , difficult breathing , swelling of periorbital sinuses with conjunctivitis , nasal and ocular discharges and diarrhea were the common signs. Mortality rate ranged from 5% to 70% in affected broiler flocks .While the examined layer flocks showed depression that may accompanied by mild respiratory signs and periorbital edema. Drop in egg production ranged from 30% to 50% in affected layer flocks with decrease in feed intake **(Table 5).**

Postmortem lesions: The postmortem finding of the examined broiler flocks showed petechial hemorrhage on breast muscle, tracheitis ranged from mild to severe, congestion in trachea, congestion in lung and congested swollen spleen, hemorrhagic congested duodenum and pancreas, enlarged bursa of fabricious and mild pericarditis **(Table 5).**

H9 avian influenza virus isolation in ECE: The H9 virus isolation trials revealed death of the embryos within 2-5 days after inoculation. Congestion of the embryos was observed **(Table 6).**

Plate haemagglutination test (HA test): Eighteen samples were tested by plate HA test giving positive result while other samples giving no heamagglutinating activity after 2nd passage and 3rd passage in ECE **(Table 6)**.

Chromatographic strip test for common antigen group A influenza: Eighteen positive hemagglutinating samples were tested by rapid chromatographic strip test; the result was 7 positive for common antigen group A influenza using this rapid test **(Table 6)**.

qrt-PCR: Real time PCR results showed 2 positive samples (12B & 19L) for H9 avian influenza. Both isolates (12B & 19L) were tested also for H5 and ND giving negative result **Table (6)** and **(Fig. 1&2).**

Conventional RT- PCR: RT- PCR result confirmed that isolate 12B was H9 AIV also confirmed that isolate 12B was free from contamination with H5 AIV and Newcastle virus **(Table 6 and Fig. 3).**

Titration of H9 isolates: Two isolates (12B & 19L) titrated giving $EID_{50}= 10^{8.5}$and 10^{8} respectively **(Table 6)**.

Pathogenicity test: Pathogenicity test was done for the H9 positive isolates in male Hyline (42day) and the result as shown in **table (7)**. In this test we observe that clinical signs started to appear at virus concentration 1/1000 with $EID_{50} =10^{5.5}$ such as whitish diarrhea, cyanosis in comb and wattle and subcutaneous hemorrhage in leg shank other than that sudden death occurred without any clinical signs. The mortality pattern was changed by changing the dose of the virus. The postmortem lesions which appeared were petechial hemorrhage on proventriculus, congested trachea and lung. Pathogenicity index (PI) was 0.8 for $EID_{50} =10^{6}$ with small dose of the virus (0.2ml) while when $EID_{50} =10^{5.5}$ given by higher doses(>0.2ml) the PI was 1.1.

Experiment result: Challenge was done with isolate 12B ($EID_{50}= 10^{8.5}$) at concentration of 1/100(EID_{50} $10^{6.5)}$ in 120 broiler chicken 21day old divided into 3 groups **(Table 8).** In this challenge we observe that sudden death occurred at day 2 (36h) post infection. Also we observed that MA5-H9 group showed higher and faster mortality than other groups **(Table 8)**. No clinical signs appeared. Postmortem lesions varied from absence of lesions to petechial hemorrhage or congested breast muscle, congested trachea, congested lung, congested duodenum, enlarged spleen, hydropericardium and sometimes bursa of fabricious showed petechial hemorrhage on its internal surface. The last 3 groups in **table (8)** are control negative which did not show any change in their conditions. Virus re-isolated from different organs screened by HA and confirmed by chromatographic strip test. HI titers as shown in **table (9)** for H9 significantly increased while HI titer for ND & H5 decreased or remained the same.

Table (1): Descriptive data of examined broiler flocks that suspected to be infected with H9avian influenza.

NO.	Locality	size of flock	Breed	Age/day	Collection date
1B	Dakahlia governorate	5000	Saso	52	14/1/2012
2B		2000	Broiler	20	15/1/2012
3B		7000	Broiler	12	18/1/2012
4B		2500	Broiler	23	19/1/2012
5B		3000	Broiler	30	21/1/2012
6B		6000	Saso	57	22/1/2012
7B		4200	Broiler	28	23/1/2012
8B		5000	Broiler	28	23/1/2012
9B		28000	Broiler	21	17/3/2012
10B		2000	Broiler	33	29/8/2012
11B		25000	Broiler	34	22/1/2013
12B		**12000**	**Broiler**	**27**	**2/12/2013**
13B		5000	Broiler	28	8/2/2014
14B		4300	Broiler	28	29/12/2014
15B		5000	Saso	53	3/1/2015
16B		2500	Broiler	24	27/1/2015
17B		15000	Broiler	38	7/2/2015
18B		4500	Broiler	37	3/9/2015
19B		6000	Broiler	21	7/9/2015
20B		5000	Broiler	33	25/9/2015
21B		5000	Saso	36	4/10/2015
22B		8500	Broiler	18	6/10/2015
23B		6500	Broiler	26	22/10/2015
24B		5000	Broiler	27	11/11/2015
25B		5200	Broiler	19	23/11/2015
26B		3000	Broiler	28	23/11/2015
27B		30000	Saso	40	11/12/2015
28B		4500	Saso	44	16/12/2015

Table (2): Descriptive data of examined layer flocks that suspected to be infected with H9 avian influenza.

NO.	Locality	size of flock	Breed	Age/day	Collection date
1L	Dakahlia governorate	4000	Layer	270	9/12/2012
2L		3000	Layer	280	22/1/2013
3L		3000	Layer	410	23/1/2013
4L		16000	Layer	249	3/2/2013
5L		3500	Layer	400	11/11/2013
6L		2000	Layer	305	19/11/2013
7L		3000	Layer	185	9/12/2013
8L		3500	Layer	156	28/12/2013
9L		5000	Layer	210	12/1/2014
10L		8000	Layer	124	15/1/2014
11L		4000	Layer	240	19/1/2014
12L		4000	Layer	160	8/2/2014
13L		3000	Layer	111	2/12/2014
14L		2000	Layer	300	5/12/2014
15L		8000	Layer	95	19/12/2014
16L		5200	Layer	210	20/12/2014
17L		3000	Layer	150	21/12/2014
18L		2000	Layer	196	15/4/2015
19L		**10000**	**Layer**	**420**	**23/4/2015**
20L		5150	Layer	180	4/5/2015
21L		3500	Layer	230	1/8/2015
22L		7500	Layer	200	3/12/2015

B=Broiler L=layer

Table (3): Sequence of oligonucleotide primers and probes used for qrt-PCR which were supplied from Metabion (Germany).

Genotype	Primer/ probe sequence (5'-3')	References
H5	**H5FH1** ACATATGACTAC CCACARTATTCA G	**Löndt *et al*., (2008)**
	H5RH1 AGACCAGCT AYC ATGATTGC	
	H5PRO [FAM]TCWACA GTGGCGAGT TCCCTAGCA[TAMRA]	
H9	**H9F** GGAAGAATTAATTATTATTGGTCGGTAC	**Ben Shabat *et al*., (2010)**
	H9R GCCACCTTTTTCAGTCTGACATT	
	H9 Probe [CY5]AACCAGGCCAGACATTGCGAGTAAGATCC[BHQ]	

F=forward R=reverse PRO. =probe

Conventional RT-PCR: Viral RNA was extracted with the Gene JET RNA Purification Kit #K0731, #K0732according to the manufacturer's instruction. The amplification took place under the following conditions: RT at 48°C for 45 min, one cycle at 94°C for 2 min, 40 cycles of heat denaturation at 94°C for 30 s, primer annealing at 57°C for H5, 60°C for H9 and 58°C for NDV for 1 min, primer extension at 68°C for 1 min and one cycle of final extension step at 68°C for 7 min in automated thermal cycler.

Table (4): Different primers used in Conventional RT- PCR to ensure that isolate 12B is free from contamination.

Target gene	Primer sequence	Amplicon length(bp)	Reference
ND	**NDF:5-**GCAGCTGCAGGGATTGTGGT-3 **NDR:5-**TCTTTGAGCAGGAGGATGTTG-3	**356 bp**	**Nanthkumar *et al.*, (2000)**
H9	**H9F:**5-GAATCCAGATCTTTCCAGAC-3 **H9R:**5-CCATACCATGGGGCAATTAG-3	**383 bp**	**WHO, (2005)**
H5	**H5F:**5-ACAAAGCTCTATCAAAACCCAAC-3 **H5R:**5-TACCCATACCAACCATCTACCAT-3	**499 bp**	**Chaharaein *et al.*, (2007)**
H9	**H9F:**5-ATCGGCTGTTAATGGAATGTGTT-3 **H9R:**5-TGGGCCTCTTGAATAGGGTAA-3	**221 bp**	**Chaharaein *et al.*, (2007)**
H5	**H5F:**5-GCCATTCCACAACATACACCC-3 **H5R:**5-CTCCCCTGCTCATTGCTATG-3	**219 bp**	**WHO, (1998)**

F=forward R=reverse

Table (5): Descriptive data of examined layer & broiler flocks in Dakahlia governorate that suspected to be infected with H9 avian influenza which gave positive result in HA test**.**

No.	Size of the flock	breed	age	Date of collection	Clinical signs	PM
2B	2000	Broiler	20	15/1/2012	Sneezing,coughing, whitich reddish diahrea	congested trachea, pneumonic lung,
3B	7000	Broiler	12	18/1/2012	Gasping, rales	Fibrinous pericarditis perihepatitis , congested trachea, pneumonic lung
5B	3000	Broiler	30	21/1/2012	Greenish diarrhea, respiratory, nervous signs	Congested intestine, enlarged spleen ,petechial hemorrhage on proventriculous
6B	6000	Saso	57	22/1/2012	Gasping, rales	Fibrinous pericarditis perihepatitis , congested trachea, pneumonic lung
8B	5000	Broiler	28	23/1/2012	Rales ,respiratory distress ,gasping	Congested enlarged kidney ,urates in ureters, congested trachea, pneumonic lung
9B	28000	Broiler	21	17/3/2012	Sneezing ,coughing, sudden death	Congested enlarged liver &spleen, congested trachea, pneumonic lung, congested duodenum
10B	2000	Broiler	33	29/8/2012	Rals,sneezing, stretched head with opened peak, whitish reddish diahrea	Caseous plug at tracheal bifurcation, fibrinous pericarditis perihepatitis, cecal coccidiosis
11B	25000	Broiler	34	22/1/2013	Gasping, rales	congested trachea, pneumonic lung
12B	**12000**	**Broiler**	**27**	**2/12/2013**	Respiratory signs, sudden death, whitish diarrhea,.	Enlarged spleen ,congested duodenum ,congested trachea, pneumonic lung, fibrinous pericarditis
15B	5000	Saso	53	3/1/2015	Sneezing, coughing, sudden death , Gasping, rales	Fibrinous pericarditis perihepatitis , congested trachea, pneumonic lung
16B	2500	Broiler	24	27/1/2015	Greenish diarrhea, respiratory, nervous signs	Congested intestine, enlarged spleen ,petechial hemorrhage on proventriculous
18B	4500	Broiler	37	3/9/2015	Gasping, rales	Fibrinous pericarditis perihepatitis , congested trachea, pneumonic lung
23B	6500	Broiler	26	22/10/2015	Respiratory signs, sudden death, whitish diarrhea.	Enlarged congested bursa, congested trachea, pneumonic lung
28B	4500	Saso	44	16/12/2015	Gasping, rales	congested trachea, pneumonic lung,
4L	16000	Layer	249	3/2/2013	40% Drop in egg production	, Congested trachea, pneumonic lung, coccidial velvety texture of intestine
17L	3000	Layer	150	21/12/2014	40% Drop in egg production	No marked signs appear
19L	**10000**	**Layer**	**420**	**23/4/2015**	50%Drop in egg production, respiratory signs	Congested trachea, pneumonic lung, congested duodenum, petechial hemorrhage on breast muscle.
21L	3500	Layer	230	1/8/2015	30% Drop in egg production	No marked signs appear

B=Broiler L=layer

Table (6): The result of plate haemagglutination, chromatographic strip test, qrt-PCR, RT-PCR and titration of positive heamagglutinating samples.

Sample NO.	ECE lesions	NO. of Passage in ECE			Plate HA Log.2	Chromato graphic strip test	qrt-PCR			RT-PCR	Titration EID_{50}
		1st	2nd	3rd			H9	H5	ND		
2B	Cong.	_	+	+	4	-	Nd	nd	nd	nd	nd
3B	Norm.	_	+	+	4	-	Nd	nd	nd	nd	nd
5B	Cong. & pet.Hg	+	+	+	4	+	-	nd	nd	nd	nd
6B	Cong.	-	+	+	4	-	Nd	nd	nd	nd	nd
8B	Norm.	-	+	+	5	+	-	nd	nd	nd	nd
9B	Norm.	+	+	+	5	+	-	nd	nd	nd	nd
10B	Cong.	-	+	+	4	-	Nd	nd	nd	nd	nd
11B	Norm.	-	+	+	5	+	-	nd	nd	nd	nd
12B	**Cong. & pet.Hg**	**+**	**+**	**+**	**5**	**+**	**+CT22.15**	**-**	**-**	**+H9 -H5 -ND**	**$10^{8.5}$**
15B	Norm.	-	+	+	5	-	nd	nd	nd	nd	nd
16B	Norm.	_	+	+	4	-	nd	nd	nd	nd	nd
18B	Cong. & pet.Hg	+	+	+	4	-	nd	nd	nd	nd	nd
23B	Norm.	_	+	+	4	-	nd	nd	nd	nd	nd
28B	Norm.	-	+	+	4	-	nd	nd	nd	nd	nd
4L	Cong.	+	+	+	5	-	nd	nd	nd	nd	nd
17L	Norm.	_	+	+	4	-	nd	nd	nd	nd	nd
19L	**Cong.**	**-**	**+**	**+**	**5**	**+**	**+CT19.57**	**-**	**-**	**nd**	**10^{8}**
21L	Cong.	+	+	+	5	+	-	nd	nd	nd	nd

L=layer B=Broiler -=negative +=positive nd=not done Cong=congestion pet.Hg=petechial hemorrhage Norm. =normal

Table (7): Pathogenicity index of two H9 isolates (12B, 19L) with EID_{50} =$10^{8.5}$ tested in male Hyline breed chicken (42days) with different concentrations and routes.

Sample code	concentration	EID_{50}	Breed	No.	Route	Mortality follow up post infection										Pathogenicity index PI
						1	2	3	4	5	6	7	8	9	10	
12B	1/10	$10^{7.5}$	H	10	I/V	-	9	1	-	-	-	-	-	-	-	**1.9**
12B	1/10	$10^{7.5}$	H	10	O/N	-	8	2	-	-	-	-	-	-	-	**1.8**
12B	1/10	$10^{6.5}$	H	10	O/N	-	2	-	1	-	1	-	-	-	-	**1.6**
12B	1/500	10^{6}	H 0.2ml	10	O/N	-	-	-	-	-	-	-	4	-	-	**0.8**
12B	1/500	10^{6}	H 0.4ml	10	O/N	-	4	3	0	2	1	-	-	-	-	**1.2**
12B	1/1000	$10^{5.5}$	H	10	O/N	1	1	0	2	1	-	-	-	-	-	**1.1**
19L	1/1000	10^{5}	H	10	O/N	-	-	-	-	-	-	-	-	-	-	**0**

I/V=intravenous O/N=oculonasal H = male Hyline breed

Table (8): Mortality in broiler (23d) groups challenged with concentration 1/100(EID_{50} =$10^{6.5}$ of isolate 12B via oculonasal route.

Group		H9 infection O/N 1/100	MA-5 Vaccination O/N twice at 8 &16d	Clone-30 vaccination O/N at 18d	No.	Mortality follow up post infection									
						1	2	3	4	5	6	7	8	9	10
1	H9	+	-	-	20	-	-	4	6	6	2	2	-	-	-
2	MA5-H9	+	+	-	20	-	-	11	7	2	-	-	-	-	-
3	Clone30-H9	+	-	+	20	-	-	5	7	5	2	1	-	-	-
4	Control	-	-	-	20	-	-	-	-	-	-	-	-	-	-
5	MA5	-	+	-	20	-	-	-	-	-	-	-	-	-	-
6	Clone30	-	-	+	20	-	-	-	-	-	-	-	-	-	-

O/N=oculonasal

Table (9): HI results of pathogenicity test and experimental challenge for H9 & H5 and ND for isolate12B in both male Hy-line layers (42day) and broiler (21-26day).

virus	Type of bird	HI Pre challenge reading							HI Post challenge reading						
		1	2	3	4	5	6	Mean	1	2	3	4	5	6	Mean
H9	H	0	0	0	0	0	0	0^{b}	7	0	8	6	7	6	5.6^{a}
	B	6	9	6	9	8	6	7.3^{b}	9	9	8	7	9	8	8.3^{a}
H5	H	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	6	4	7	4	5	0	4.3^{a}	0	6	6	3	3	0	3^{b}
ND	H	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	B	3	4	3	5	4	4	3.8^{a}	0	0	3	0	0	2	0.8^{b}

The row has the different letter is significantly difference at P≤0.05

H= male Hy-line breed chickens aged 42 days B= broiler chickens aged 21 days

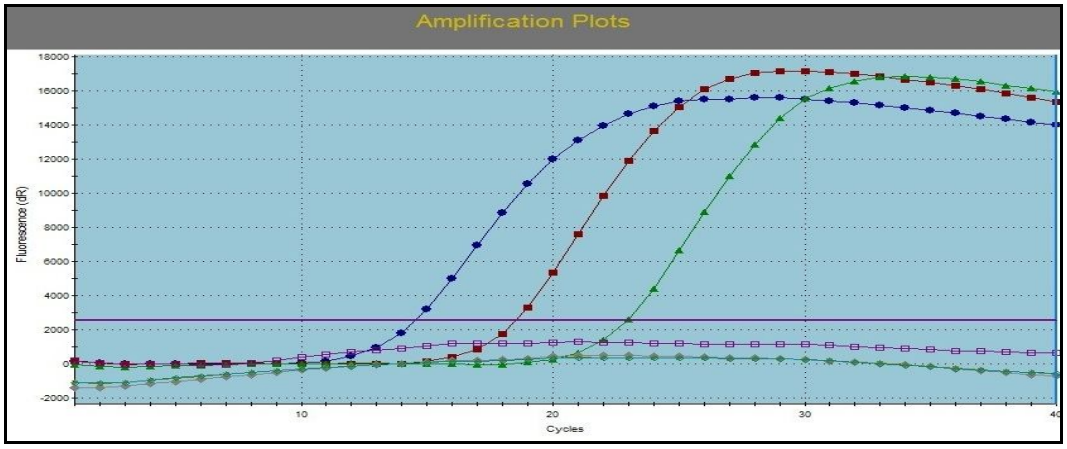

Figure (1): Amplification curve for AIV- H9 gene qrt-PCR isolate 19L &isolate12B.the CT value 19.75 & 22.15.

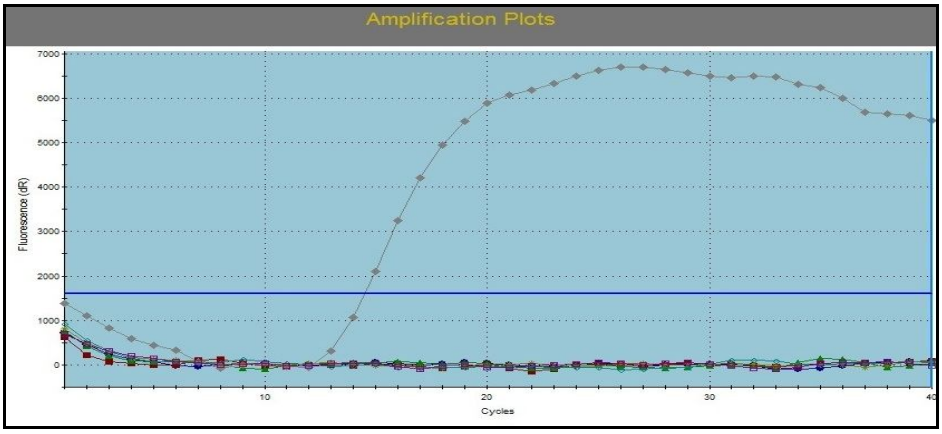

Figure (2): Amplification curve for AIV- H5 gene qrt-PCR s isolate 19L &isolate12B.the control curve only appear.

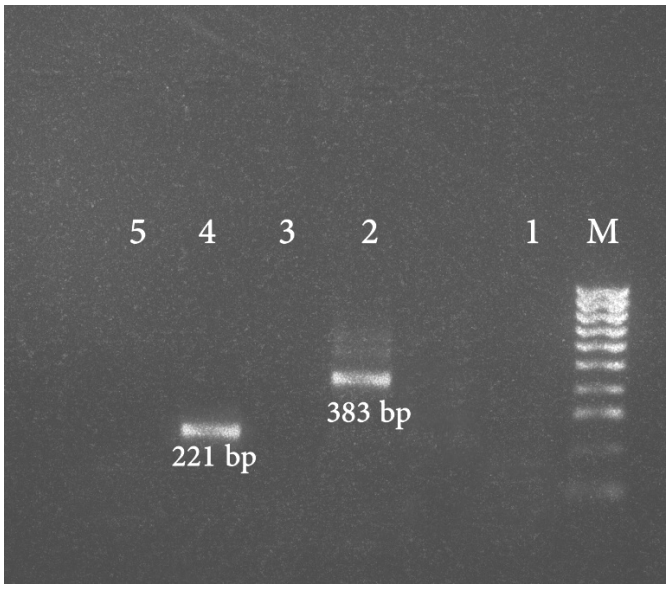

Figure (3): Agarose gel electrophoresis of the RT-PCR product for isolate.12B. Lane M: marker 100 bp plus, lane 1=-ND, lane 2=positive amplification of 383 bp fragment of H9 gene, lane 3=-H5 gene, lane 4= positive amplification of 221bp fragment of H9 gene, lane5=-H5gene.

Acknowledgments

Authors acknowledge Professor Dr. kamel Abou-Elazm and his private laboratory for his kind help and for performing RT –PCR.

DISCUSSION

Unlike known for H9 that it has no clinical signs or postmortem lesions **(Bernard *et al.,* 2001 and Capua and Alexander, 2004)** we found that depression, reduction of feed intake but not complete cessation or off food and the birds showed some sort of laziness are common when the infection is not sever ,while in severe infection, we found that birds showed respiratory symptoms as sneezing, coughing, tracheal rales, swelling of periorbital sinuses with conjunctivitis, whitish diarrhea as stated by **Nili and Asasi ,2002 and Seifi *et al,*. 2010**. While in extreme case of disease in case of higher doses of virus it showed sudden death without any signs after less than 48h of infection. Regarding field cases, mortality rate differed widely as stated by **Capua and Terregino, (2009)** and our observation agreed with what stated by them and increased slightly than 50% in field cases and in our experimental challenge.

In our field study we approved that co-infection with any other viral or bacterial pathogen exacerbate disease condition with H9 infection which increasing mortality rate up to 80% **(Kishida *et al.,* 2004 and Haqshenas *et al.,* 2005).** The reason for increasing the pathogenicity of H9 could be explained by various hypotheses, increasing cleavage of H9 by bacterial proteases **(Tashiro *et al.,* 1987)** or stimulation of host cell to secret more proteases or destruction of endogenous cell protease inhibitors found in respiratory tract responsible for inhibition of virus cell entry and viral uncoating **(Mancini *et al.,* 2005).** It was assured that signs of viral disease vary according to dose of the virus and its concentration even with low pathogenic H9 avian influenza **(Bernard *et al.,* 2001 and OIE 2012)** as in this experiment mortality reached 100% in higher doses and higher concentration of AIV-H9. In our experiment the Pathogenicity index ranged from 1.9 in higher doses and concentrations to 0.8 in lower doses.

Our observation agreed with what informed by **Mosleh *et al.,* (2008)** that the tissue tropism of H9 is confined to respiratory, urinary, lymphoid and digestive systems which appeared obviously in our experimental challenge on postmortem lesions on birds in the form of congested trachea and lung, hemorrhage in small intestine especially duodenum, hemorrhage in the pancreas, swollen kidneys, enlarged spleen, pinpoint hemorrhage in bursa of fabricious, also observation of cyanosis of wattles and combs of few birds was matched with their observation. Those observations were similar to lesions reported in naturally infected chickens with H9N2 **(Nili and Asasi, 2002).**

In our experimental challenge we agreed with **Haghighat-Jahromi *et al.,* (2008)** who showed that the infectious bronchitis (IB) live vaccine exacerbates the manifestation of experimental H9 infection in broiler chicken that we found the faster mortality occurred in group MA5-H9 which was vaccinated by live strain of IB and infected with H9. The reasons of this condition were explained by **Cook *et al.,* (1976)** that IB virus could aggravate ciliostasis in the host's ciliated airways which give the opportunity for the other related pathogens to be pathogenic. **Liu *et al.,* (1995) and NG and Liu, (2000)** also added that IB co-infection may have provided the enzymes and enhanced H9 pathogenicity in the affected flocks and attributed the reason for that to a trypsin-like serine protease domain is encoded by corona virus (IBV).

Inoculation in SPF ECE via allantoic cavity was the method we adopt for isolation of H9 AIV. Recording death of embryo within 2-5 days with some lesions on it such as

congestion and slight hemorrhage agreed with what stated by **Vasfi Marandi and Bozorgmehrifard, (2002) ; Swayne and Halvorson, (2003) and Haghighat-Jahromi *et al.,* (2008).**

As recommended by **OIE (2014)** for diagnosis of AIV and similar to **Bernard *et al .,* (2001)** ; **Capua and Terregino, (2009) and Abdel-Moneim *et al.,* (2012)** , plate HA test was the first screening test for allantoic fluid of 50 samples-under study in this research- which resulted in 18 positive hemagglutinating samples that reduced the circle of suspicion to avian influenza and Newcastle viruses and giving us an idea about concentration of virus and the need for 2nd and 3rd passage for the suspected isolates however 2nd passage is recommended even with 1st passaged negative hemagglutinating samples.

chromatographic strip test for common antigen of influenza type A is a rapid detection method for avian influenza (**Cui and Tong, 2008 and Abdel-Moneim *et al.,* 2012)**. In our investigation we used it as our guide to keep our feet on the right path by detecting 7samples out of 18 to be suspected AIV. Conventional RT-PCR detect two subtypes (H5 and H9) of AIV and ND virus which was conducted before by **Spackman *et al.,* (2003) and Xie *et al.,* (2006)**. In our study this method also was successfully amplified the targeted genes with expected sizes.

In our study we agreed with **Haghighat-Jahromi *et al.,* (2008) and Afifi *et al.,* (2012)** that under field condition, infection mostly occurs with more than one virus so conventional RT-PCR for different viruses at the same time or multiplex PCR technique may offer a substitutional method for immediate detection of different viruses at once.

Our results agreed with **Motamed *et al.,* (2013)** in that some H9 isolates has low pathogenic effect at $EID_{50} = 10^{5.5}$ and 10^{6} but the later restricted with the dose 0.2ml while with higher dose and also with $EID_{50} = 10^{6.5}$ it has intermediate pathogenicity on contrary to the findings of **Aslam *et al.,* (2015)**. The same isolate was highly pathogenic with $EID_{50} = 10^{7.5}$ on contrary to what stated by **Noroozian *et al.,* (2007)**.

CONCLUSION & RECOMMENDATION

In conclusion, our results confirmed the continuous circulation of the AIV H9 in chicken flocks in Dakahlia governorate which complicate the respiratory problems in affected flocks. From 50 examined flocks 18 samples were positive HA, 7 out of 18 were positive by chromatographic strip test, 2 isolates were positive PCR without any contamination with H5 and ND viruses. Pathogenicity index (PI) was 0.8 with EID_{50} $10^{5.5}$. Higher doses with low EID_{50} increase PI. IB live strain vaccine increase pathogenicity of H9. Continuous monitoring and detection of gene mutations in AIV- H9 are recommended.

REFERENCES

Aamir, U. B.; Wernery, U.; Ilyushina, N. and Webster, R. G. (2007): Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology 361, 45–55.

Abddel-Moneim, A. ; Afifi, M. and El-Kady, M.(2012) : Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt . Virology journal 2012, 9:173. Doi:10.1186/1743-422x-9-173.

Afifi, M.; El-Kady, M.; Zoelfakar, S. and Abddel-Moneim, A. (2012): Serological surveillance reveals widespread influenza A H7 and H9 subtypes among chicken flocks in Egypt . Trop Anim Health Prod. DOI 10.1007/s11250-012-0243-9.

Aslam, R.; Aslam, A.; Tipu, Y.; Nazir, J.; Ghafoor, A. and Fatima, S. (2015): Histopathological and immunohistochemical studies for thePathogenesis of a low pathogenicity h9 avian influenza virus in experimentally infected commercial broilers. J. Anim. Plant Sci. 25(1): pp: 45-52.

Beard, C.W. (1989): Serological procedures. In a laboratory manual for the isolation and identification of avian pathogens. 3rd edition. Edited by Purchase HG,Arp L.H., Domermuth C.H., Pearson J.E. Dubuque, Iowa: Kendall/Hunt Publishing Company; 192–200.

Ben Shabat, M.; Meir, R.; Haddas, R.; Lapin, E.; Shkoda, I.; Raibstein, I.; Perk, S. and Davidson, I. (2010): Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J Virol Methods.; 168 (1-2):72-7.

Bernard, N. F.; Peter, M.; Howley, M.D.; Diane, E.; Griffin, E.; Robert, A.;Lamb E.; Malcolm, A.; Martin, M.D.; Bernard, R.; Stephen, E.; Straus, M.D.; David, M. and Knipe, E.(2001): Fields - Virology 4th Ed. (August 2001). Lippincott Williams & Wilkins Publishers. (Two Volumes)

Butt, K. M.;Smith, G. J.; Chen, H.;Zhang, L. J.; Leung, Y. H.; Xu, K.; MLim, W.; Webster, R. G. and Yuen, K. Y. (2005): Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43, 5760–5767.

Capua, I. and Alexander, D.J. (2004): Avian influenza: recent developments. Avian Pathol., 33: 393–404.

Capua,I. and Terregino,C.(2009): Clinical Traits and Pathology of Avian Influenza Infections, Guidelines for Farm Visit and Differential Diagnosis. Avian Influenza and Newcastle Disease A Field and Laboratory Manual ed. Ilaria Capua and Dennis J. Alexander eds Springer-Verlag Italia 2009. pp. 45-72.

Chaharaein, B.; Omar, A.R.; Aini, I.; Yusoff, K. and Hassan, S.S. (2007): RT-Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription-polymerase chain reaction. Microbiological Research 164 :174—179.

Cook, J. K. A.; Darbyshire, J. H. and Peters, R. W. (1976): The use of chicken tracheal organ cultures for the isolation of avian infectious bronchitis virus. Archi. Virol. 50, 109-118.

Cui, S. and Tong, G. (2008): A chromatographic strip test for rapid detection of one lineage of the H5 subtype of highly pathogenic avian influenza. J Vet Diagn Invest 20:567–571.

Gou, Y. J.; Xie, J. P.; Wang, M.; Dong, J. ;Gou, J. F.; Zhang, H. and Wu, K. Y.(2000): A strain of influenza A H9N2 virus repeatedly isolated from a human population in China. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 14:209–212.

Haghighat -Jahromi, M.; Asasi, K. ; Nili, H.; Dadras, H. and Shoshtari , A. H.

(2008): Co-infection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Archi. virol. 153, 651-655.

Halvorson, D.A. (2002): The control of H5 or H7 mildly pathogenic avian influenza: a role for inactivated vaccine. Avian Pathol 31:5-12.

Haqshenas, G.; Asasi, K. and Akrami, H. (2005): Isolation and molecular characterization of infectious bronchitis virus, isolate Shiraz 3. IBV, by RT-PCR and restriction enzyme analysis. Iranian J. Vet. Res. 6, 9-15.

Karimi-Madab, M.; Ansari-Lari, M.; Asasi, K. and Nili, H. (2010): Risk factors for detection of bronchial casts, most frequently seen in endemic H9N2 avian influenza infection, in poultry flocks in Iran. Prev. Vet. Med., 95 : 275-280.

Kishida, N.; Sakoda, Y. ;Eto, M. ; Sunaga, Y. and Kida, H. (2004): Co-infection staphylococcus aureus or Haemophilus paragallinarum exacerbates H9 N2 influenza A virus infection in chicken. Arch. Virol. 149, 2095-2104.

Liu, D. X.; Brierley, I. and Brown, T. D. (1995): Identification of a trypsin-like serine proteinase domain encoded by ORF 1a of the coronavirus IBV. Adv. Exp. Med. Biol.380, 405-411.

Lǒndt, B.Z.; Nunez, N.; Banks, J.; Nili, H.; Johnson, L.K. and Alexander, D.J. (2008): Pathogenesis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (Anas platyrhynchos) infected experimentally. Avian Pathology (December 2008) 37(6), 619_627.

Mancini, D.A.P.; Mendonca, R.M.Z.; Dias, A.L.F.; Mendonca, R.Z. and Pinto, J.R. (2005): Co-infection between influenza virus and flagellated bacteria. Rev.inst. Meb. Trop. Sao Paolo; 47:275-280.

Mosleh, N.; Dadras, H. and Mohammadi, A. (2008): Evaluation of H9N2 avian influenza virus dissemination in various organs of experimentally infected broiler chickens using RT-PCR. Iranian Journal of Veterinary Research, Shiraz University, Vol. 10.pp.12-20. No. 1, Ser. No. 26, 2009.

Motamed, N.; Mayahi, M.; Seifi, M. R. and Jafari, R. A. (2013): Effect of infectious bursal disease virus on pathogenicity of avian influenza virus subtype H9N2 in broiler chicks . 2013 Academic Journals Vol. 5(10), pp. 276-280, October, 2013 .DOI 10.5897/JVMAH12.052.

Nanthakumar ,T.; Kataria, R.S.; Tiwari, A.K.; Butchaiah, G. and Kataria, J.M. (2000): Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. Veterinary Research Communications 24 : 275-286.

NG, L. F. P. and Liu, D. X. (2000): Futher characterization of the coronavirus infectious bronchitis virus C-like proteinase and determination of a new cleavage site. Virology 272, 27-39.

Nili, H. and Asasi, K. (2002): Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran, Avian Pathology, 31:3, 247-252, DOI: 10.1080/03079450220136567.

Noroozian, H.; Vasfi Marandi, M. and Razazian, M. (2007): Detection of avian influenza virus of H9 subtype in

the faeces of experimentally and naturally infected chickens by reverse transcription-polymerase chain reaction. ACTA VET. BRNO 2007, 76: 405-413; doi:10.2754/avb200776030405.

OIE (2012): Version adopted by the World Assembly of Delegates of the OIE in May 2012. OIE Terrestrial Manual 2012. Chapter 2.3.4.pp1-17. http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

OIE Terrestrial Manual avian infuenza(2014): Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Reed, L.J. and Muench, H. (1938): A simple method for estimating fifty percent endpoints. Am.J. Hyg. 27:493-497.

Seifi, S; Asasi, K. and Mohammadi, A. (2010): Natural co-infection caused by avian influenza H9 subtype and infectious bronchitis viruses in broiler chicken farms . Vet. arhiv 80, 269-281, 2010.

Sorrell, E.M.;Wan, H.; Araya, Y.; Song, H. and Perez, D.R. (2009): Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc Natl Acad Sci U S A 106: 7565–7570.

Spackman, E.; Senne, D.A.; Bulaga, L.L.; Trock, S. and Suarez, D.L. (2003): Development of multiplex real-time RT-PCR as a diagnostic tool for avian influenza. Avian Dis;47:1087–90.

Steel, R.G. and Torrie, J.H. (1980): Principles and procedures of statistics. MC Graw-Hill puplishers new York, 2nd edition.

Swayne, D. E. and Halvorson, D. A. (2003): Influenza. In: Disease of Poultry. Disease of Poultry. (Saif Y. M., H. J . Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, D. E. Swayne, Eds.). 11th ed., Ames Iowa State Press. pp. 135-160.

Tashiro, M.; Ciborowski, P.; klenk, H.D.; pulverer, G. and Rott, R. (1987): Role of staphylococcus protease in the development of influenza pneumonia. Nature; 325:536-537.

Tong,S.;Zhu,X.;Li,Y.;Shi,M.;Zhang,J.;Bourgeois,M.;Yang,H.;Chen,X.;Recuenco,S.;Gomez,J.;Chen,L.;Johnson,A.;Tao,Y.;Dreyfus,C.;Yu,W.;Mcbride,R.; Carney,P.J.;Gilbert,A.T.;Chang,J.;Guo,Z.;Davis,C.T.;Paulson,J.C.;Steven,J.;Rupprecht,C.E.;Holmes,E.C.;Wilson,I.A. and Donis, R.O.(2013) : New world bats harbor diverse influenza A viruses . PLOS pathogen;9(10):e1003657.

Vasfi- Marandi, M. and Bozorgmehrifard, M.H. (2002): isolation of H9N2 subtypes of avian influenza viruses during an outbreak in iran. Iranian Biomedical Journal; 6, 13-17.

Villegas, P. and Purchase, H.G. (1989): Titration of biological suspensions. In: Purchase, H.G.; ARP, L.H.; Domermuth, C.H. and Pearson, J.E. A laboratory manual for isolation and identification of avian pathogens, 3rd ed. American Association of Avian Pathologists, Kennet Square,Penn.PP. p.86.191.

Wan, H. and Perez, D.R. (2007): Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human

airway epithelial cells. J Virol 2007, 81:5181-5191.

WHO Geneva (2005): Recommended laboratory tests to identify avian influenza A virus in specimens from humanp. 1-7

WHO H5 reference lab(1998): Network modified by Yuen et al 1998 cited by Taher , M.M. ; Elhurn A.El-Ebjary, Soad, M.Soliman, Ali,A.M.; lumia , M. ; Omar, Manal Awad; Hyam Farouk , Nassif , S.A. Global veterinaria 315):390-394,2009.lancet , 351:467-471,1998

Xie, Z.; Pang,Y.S.; Liu,J.; Deng,X.; Tang,X. and Sun,J. (2006):A multiplex RT-PCR for detection of type A influenza virus and differentiation of avian H5, H7 and H9 hemagglutinin subtypes. Mol Cell Probes; 20:2459.

Xu, K. M.; Li, K. S.; Smith, G. J.; Li, J. W.; Tai, H.; Zhang, J. X.; Webster,R. G.; Peiris, J. S.; Chen, H. and Guan, Y. (2007): Evolution andmolecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000 to 2005. J Virol 81, 2635–2645.

Zhao, M.; Liu, Q.; Sun, Q.; Zhang, W.; Zhao, G.; Gu, M.; Wang, X.; Hu, S.; Liu, X. and Liu, X. (2013): Full genome sequence of a natural reassortant H9N2 avian influenza virus isolated from domestic ducks in Jiangsu Province, China. Genome Announce. 1(4): e0046313. doi: 10.1128/genome A. 00463.

الملخص العربى

دراسات على فيروس اتش-٩ لانفلونزا الطيورفي محافظة الدقهلية

اسماء احمد الزيات*، عبدالفتاح حمدي العدل**، حنان علي فهمي***، هاني فوزي اللقاني****

*معهد بحوث صحة الحيوان فرع المنصورة
** قسم امراض الدواجن جامعة المنصورة
***معهد بحوث صحة الحيوان بالدقي
****قسم امراض الدواجن جامعة دمنهور

نظرا للتوسع الكبير في تربية الطيوروزيادة نسبة الاصابة بالامراض الفيروسية خاصة الانفلونزا بمختلف عتراتها فقد تم في هذا البحث عمل دراسة على عترة الاتش-٩ في محافظة الدقهلية عن طريق تجميع عدد ٥٠ عينة بطريقة عشوائية خلال اربع سنوات واجريت لها الاختبارات اللازمة لعزل الفيروس في اجنة بيض الدجاج وقد تم عزل عدد ٢معزولة في عام ٢٠١٣و٢٠١٥ وتاكيدهم باستخدام اختبار التلازن الدموي والاختبار السريع وسلسلة تفاعل البلمرة الكمي حيث اظهر الاخير قيم ٢٢،١٥ -١٩،٧٥ وبنفس الاختبار تم التاكد من خلو المعزولات من فيروس النيوكاسل و فيروس الانفلونزا عترة الاتش -٥ وقد تم عمل معايرة لكل من المعزولتين باستخدام اجنة بيض الدجاج لحساب تركيز الفيروس في المعزولة اللازم لعدوى ٥٠% من الاجنة حيث وجد في كلا العينتين ١٠ $^{٨،٥}$ كما تم دراسة الضراوة للمعزولات وجد انه ٠،٨ ولنفس المعزولة الاخيرة تم ملاحظة نسب الوفيات التي تحدث عند تزامن التحصين مع الاصابة بفيروس الاتش -٩ وجد ان نسب الوفيات تتسارع .

ومن خلال ملاحظة الاعراض الاكلينيكية على الطيور وجد انها تعاني من نقص في استهلاك الغذاء مع بعض الاعراض التنفسية كالعطس والافرازات الانفية و الاسهال الابيض كما وجد في الصفة التشريحية احمرار في القصبة الهوائية والرئة و نقط نزفية صغيرة على البنكرياس واحمرار في الامعاء خاصة الاثنى عشر ووجد في غدة فابريشيوس نقط نزفية صغيرة مع زيادة بسيطة في الحجم و زيادة حجم الطحال وتغير لونه.

الخلاصة: ان فيروس الانفلونزا عترة الاتش٩ اخذ في الانتشار والزيادة والتحور الدائم كما انه يصيب الدجاج البياض واللحم وان ضراوته تزداد بزيادة الجرعة وزيادة تركيز الفيروس كما انها تزداد بتزامن الاصابات البكتيرية والفيروسية وانه يؤثر سلبا على استجابة الطائر للتحصينات المختلفة وان تزامن التحصين بالعترة الحية لفيروس الالتهاب الشعبي الوبائي للطيور مع الاصابة يؤدي الى زيادة نسبة النافق وانه يلزم استكمال دراسته لمعرفة اي تحور قد يؤدي الى تحول الفيروس الى عترة شديدة الضراوة او تحوره بطريقة تؤدي الى انتقال الاصابة من انسان الى انسان.