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Research Article
Pathological and Molecular Studies on Avian Influenza Virus (H9N2) in Broilers

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Abstract

Background and Objective: Avian influenza viruses (AIV) subtype H9 is a low pathogenic virus that usually causes low mortality in the broiler birds. In this study, serotype H9N2 was screened in broiler farms showing mild respiratory manifestations in Dakahlia province, Egypt during the period from October, 2014 to January, 2015. **Materials and Methods:** H9N2 virus was isolated from only 4 broiler farms out of 25 by real time polymerase chain reaction (RT-PCR). Phylogenetic analysis of the haemagglutinin gene (HA) showed that the isolates were grouped in Quail/Hong Kong/G1/97 lineage-strains similar to the one circulating in the Middle East. Specific pathogenic free-embryonated chicken embryo (SPF-ECE) was inoculated with PCR-positively infected materials on allantoic cavity. Histopathological examination was followed by immunohistochemistry staining of H9 virus in formalin fixed paraffin-embedded and frozen tissue specimens. **Results:** By immunohistochemistry, the virus was stained in trachea, lungs and kidneys of broiler chickens, in liver and intestine of inoculated chicken embryos. **Conclusion:** Site of replication of the H9N2 virus in tissues differed in broilers from that in embryos. However, the virus was low pathogenic in nature, the use of biosecurity measures by poultry farmers in Egypt is recommended to avoid outbreaks.

Key words: Avian influenza, H9N2, broiler, immunohistochemistry, immunofluorescent staining

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Avian influenza viruses (AIV) are placed in the family of Orthomyxoviridae having three influenza genera known as A, B and C on the bases of antigenic difference of the NP and M1 protein[1]. Among these genera, only influenza A viruses were further classified into different subtypes. This classification was based on antigenicity of two transmembrane glycoproteins on the surface of virus which were named haemagglutinin (H/HA) and neuraminidase (N on NA)[2]. Thus viruses were separated into 16 haemagglutinin (H1 to H16) and 9 neuraminidase (N1 to N9) antigenic subtypes[3]. The influenza virus A can produce two different types of diseases on bases of their virulence and pathogenicity viz., highly pathogenic avian influenza virus (HPAIV) and low pathogenic avian influenza virus (LPAIV). Although, AIV serotype H9N2 does not belong to HPAIV, it may cause severe infection in broilers[4]. The virus also was capable of inducing severe respiratory tract infections in immunosuppressed young chicks with high mortality[5]. The infection was widely distributed in commercial poultry and migratory wild/shore birds while aquatic birds especially ducks, act as a natural host for the virus[6]. The virus can be transmitted from these natural hosts to highly prone species such as chickens and turkeys[7]. In natural reservoirs, the virus was considered non- pathogenic and replicated in intestinal tract without evident clinical signs[8]. Immunohistochemical staining showed that lungs and kidneys were the two main target organs for viral replication and infection persisted in kidneys for longer time[9,10]. In recent years, H9N2 virus has attained a great importance because of its panzootic outbreaks[11]. The frequency of these outbreaks was increasing noticeably in Iran, Pakistan and United Arab Emirates[5,12-14]. At the present there is a meager and insufficient data on the pathogenicity of H9N2 viruses. This work was designed to (1) Characterize the antigenic and molecular properties of subtype H9N2 isolates in Dakahlia province, Egypt, (2) Define genetic and phylogenetic relationships with other AIVs and (3) Determine the most affected tissues in broiler chicken and inoculated chicken embryos using avidin-biotin peroxidase (ABP) and immunofluorescent (IF) staining.

MATERIALS AND METHODS

Chickens and specimens collection: Twenty five broiler farms in Dakahlia province, Egypt showed respiratory symptoms during the period from October, 2014 to January, 2015. All flocks had been vaccinated with H120 and MA5+Clone30, ILT vaccine (Shering plough) and H5N1 vaccine. A representative number of sick broiler chickens were selected from all farms. Tissue samples from lungs and tracheas of each affected flock were polled then held at -20°C until been processed for real time polymerase chain reaction (RT-PCR), indirect IF and egg inoculation. Clinical signs of sick broilers and gross lesions of necropsied birds were recorded in positive farms for AIV H9N2 by RT-PCR. Other tissue specimens from lungs, tracheas, kidneys, spleen and liver of positive broilers for AIV H9N2 by RT-PCR were immediately fixed in 10% neutral buffered formalin for routine histopathology, ABP and IF staining.

Virus screening with RT-PCR: Viral RNA was extracted by using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions directly from the supernatants of 10% w/v sample suspensions. The extracted viral RNA was screened for the presence of AIV using RT-PCR. The reaction was performed with Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA), using specific primers and probe (Table 1) according to the previous reports of Adzhar *et al*.[15] and Callison *et al*.[16]. PCR Master Mix for AIV was done according to QuantiTech probe RT-PCR kit handbook (January, 2008).

Agarose gel electrophoresis: Amplification products were analyzed in 1.5% agarose gel. The predicted size of RT-PCR product was about 1700 bp.

DNA purification: The RT-PCR products were cut from the gel and purified using the QIAquick gel extraction kit (Qiagen Inc. Valencia CA) according to the manufacturer's protocol.

Sequencing reaction: Purified RT-PCR products were sequenced in a forward direction using forward AIV primer,

Table 1: List of primers used in RRT-PCR

Virus	Gene		
AI	M	Forward	5-AGATGAGTCTTCTAA CCGAGGTCG-3
		Reverse	5-TGCAAAAACATCTTC AAGTCTCTG-3
		Probe	5-FAM-TCAGGCCCC CTCAAAGCCGA-TAMRA-3
	H9	Forward	5-GGAAGAATTAATTATTATTGGTCGGTAC-3
		Reverse	5-GCCACCTTTTTCAGTCTGACATT-3
		Probe	5-FAM-AACCAGGCCAGACATTGCGAGTAAGATCC-TAMRA-3

Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster city, CA) and an Applied Biosystems 3130 genetic analyzer (ABI, USA) according to the manufacture instructions.

Phylogenetic analysis: To identify the Egyptian H9 isolates, sequences of the haemagglutinin(HA) gene of the Egyptian H9 isolates were compared with published H9 sequences deposited in the GenBank database using a BLAST search via the National Center of Biotechnology Information (USA). Sequence identities by BLAST analysis were included in alignment and phylogenetic construction. A phylogenetic tree of the nucleotide sequences was constructed using MEGA version 4[17]. A comparative analysis of HA gene sequences was performed using the CLUSTALW Multiple Sequence Alignment Program, version 1.83[18].

Egg inoculation: Eggs were inoculated with 0.2 mL of prepared RT-PCR positive samples to AIV H9N2 into the allantoic cavity of 9-11 days-old specific pathogenic free-embryonated chicken embryos (SPF-ECE). The inoculated eggs were incubated horizontally with the site of inoculation uppermost at 37°C for 6 days with daily examination. Embryos which died within the first 24 h post-inoculation (PI) were discarded and considered as nonspecific deaths. Selected organs (lungs, liver, kidneys and small intestine) were collected from dead embryos 5 days PI and fixed in 10% buffered formalin solution for hematoxylin and eosin (HE) and ABP staining.

Histopathological examination: All formalin-fixed tissue specimens from positive broiler chickens and embryonic tissues were routinely processed. Paraffin sections of 5 µm thickness were cut and stained with HE[19]. Histological changes were examined by light microscopy.

Avidin-biotin peroxidase (ABP) test for detection of H9N2: For detection of H9N2, ABP was applied to formalin-fixed, paraffin embedded tissue sections (trachea, lungs, kidneys) from positive birds for H9N2 by RT-PCR in addition to the selected fixed paraffin embedded embryonic tissue samples. After dewaxing and dehydration, blocking steps in the procedure included incubation of the slides with 3% H_2O_2. Avidin and biotin-labeled peroxidase antibody against H9N2 virus was applied to the slides then incubated at room temperature for 1 h with humidity. The slides were washed three times (5 min each) in a bath of phosphate buffered saline (PBS), pH 7.2. Anti-rabbit secondary antibody of 1:200 dilutions in PBS was then added to the slides and incubated for 1 h at room temperature with humidity then the slides were washed with PBS. The final reaction was achieved by incubating the sections with 0.5% 3, 3-Diaminobenzidine and examined by light microscope. Control negative slides were prepared by incubating sections with antibody against H5N1.

Indirect immunofluorescent (IF) technique: Rabbit hyperimmune serum against H9N2 was applied to the formalin-fixed paraffin embedded tissue samples (trachea, lungs, liver), frozen samples (trachea and lungs) from positive birds for H9N2 by RT-PCR and selected formalin-fixed paraffin embedded embryonic tissue samples. The slides were incubated at 37°C for 1 h with humidity then washed 3 times (5 min each) in a bath of PBS, pH 7.2. Anti-rabbit FITC conjugate with 1:200 dilution was applied on each slide for 30 min in a dark humidified chamber. After, washing, the slides were mounted with 50% glycerol in PBS and examined by a fluorescence microscope. Control negative slides were simultaneously prepared by incubating sections with primary antibody against H5N1.

RESULTS

RT-PCR, gene sequencing, amino acids identity and phylogenetic analysis: The pooled frozen trachea and lung samples from each suspected flock were screened for H9N2 with RT-PCR. Four broiler farms out of 25 were positive for H9N2 by RT-PCR as shown in Fig. 1. One screened positive sample was amplified by RT-PCR using specific primers for haemagglutinin gene (HA). Amplification product was obtained at 1700 bp. Amino acid sequences were determined and compared among each other and with other AIV strains published in the GenBank database Fig. 2. Phylogenetic analysis of the two positive samples isolated from Dakahlia province showed that they were H9N2. Phylogenetic tree for the positive sample with related strains based on the HA gene amino acids sequence was demonstrated in Fig. 3. Phylogenetic analysis of the HA gene showed that the isolates from Egypt were grouped in Quail/Hong Kong/G1/97 lineage-strains similar to the one circulating in the Middle East. The Egyptian H9N2 AIVs were located with Israeli ones in characteristic cluster among G1 lineage. Egyptian H9N2 AIVs lacked multiple basic amino acids at the cleavage sites and were regarded as viruses of low pathogenicity.

Clinical signs and gross lesions: Most of the affected birds showed slight depression with low intake of feed and water, besides, gasping, coughing, sneezing and tracheal rales. The

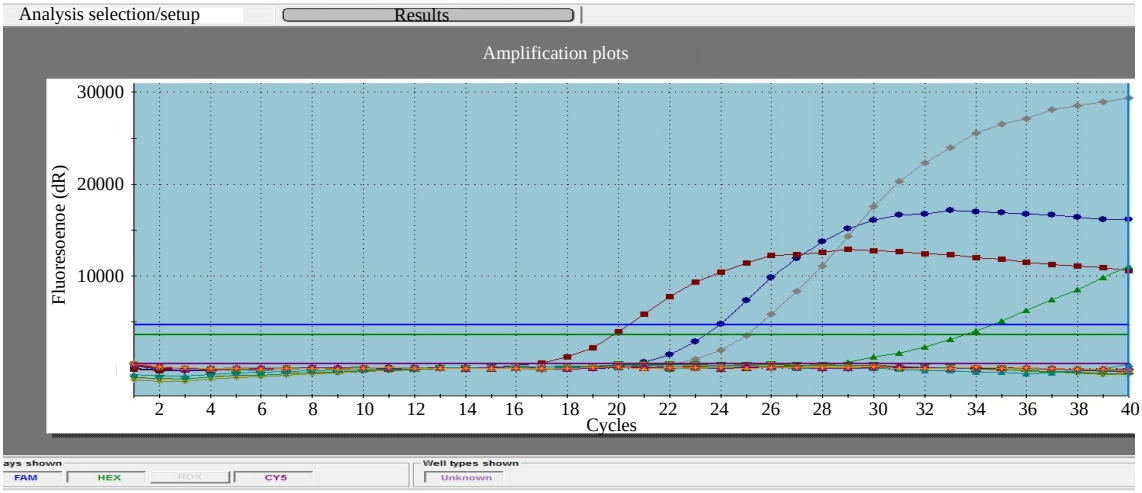

Fig. 1: Amplification curves of two H9N2 samples using RRT- PCR

Percent identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
1		98.3	98.6	98.2	98.3	99.4	98.0	98.3	98.6	97.7	97.8	99.0	98.6	97.6	98.3	98.8	98.8	98.7	97.9	98.8	98.5	90.9	96.7	89.4	90.6	82.9	98.5	88.7	88.8	88.6	1 A/chicken/Egypt/114915V/2011
2	1.7		98.0	98.8	99.1	98.6	97.2	97.6	97.5	97.0	97.8	97.8	97.9	96.9	97.5	97.8	97.8	97.7	98.6	98.1	99.1	91.0	96.8	89.5	90.9	83.4	99.1	88.4	89.4	89.9	2 A/chicken/Egypt/114940v/2011
3	1.4	2.0		98.1	97.9	98.8	98.2	98.6	97.8	98.1	97.5	98.3	98.9	97.8	98.5	98.1	98.1	98.0	97.5	99.3	98.1	90.8	96.7	89.7	90.8	83.4	98.1	88.2	88.6	88.6	3 A/chicken/Egypt/115617V/2011
4	1.8	1.2	1.9		99.3	98.4	97.2	98.5	97.5	96.9	97.7	97.8	97.8	96.9	97.5	97.7	97.7	97.6	98.6	98.0	98.9	90.6	96.6	89.2	90.6	83.1	98.9	87.8	89.3	89.1	4 A/chicken/Egypt/12186F-9/2012
5	1.7	0.9	2.1	0.7		98.5	97.2	97.6	97.5	97.0	97.8	97.8	97.9	96.9	97.5	97.8	97.8	97.7	98.8	98.1	99.1	90.8	96.7	89.4	90.9	83.2	99.1	87.9	89.4	89.2	5 A/chicken/Egypt/1226B/2012
6	0.6	1.4	1.2	1.6	1.6		98.3	98.6	98.8	98.0	97.8	99.1	99.0	97.9	98.6	99.0	99.0	98.9	98.1	99.1	98.8	91.0	97.0	89.5	91.0	83.1	98.8	88.9	88.9	88.9	6 A/chicken/Egypt/A2-D/2011
7	2.1	2.8	1.8	2.9	2.8	1.7		98.2	97.3	97.4	97.0	97.7	99.0	97.8	98.2	97.5	97.5	97.4	96.9	98.5	97.5	90.0	96.0	88.9	90.2	82.6	97.5	87.6	87.9	87.7	7 A/chicken/Egypt/D4907A/2012
8	1.7	2.5	1.4	2.5	2.5	1.4	1.8		97.7	97.8	97.4	98.2	98.8	97.7	99.8	97.9	97.9	97.8	97.2	98.8	97.9	90.5	96.1	89.4	90.5	83.1	97.9	88.1	88.3	88.1	8 A/chicken/Egypt/D5490B/2012
9	1.4	2.5	2.2	2.6	2.5	1.2	2.8	2.4		97.3	96.9	98.5	98.0	96.9	97.6	99.8	99.8	99.6	97.5	98.2	97.8	90.3	96.2	89.1	90.3	82.4	97.8	88.9	88.2	88.0	9 A/chicken/Egypt/D7436C/2013
10	2.3	3.1	1.9	3.2	3.1	2.0	2.6	2.3	2.8		96.6	97.7	98.1	97.3	97.8	97.4	97.4	97.3	96.7	98.4	97.3	89.9	95.8	88.6	90.3	82.7	97.3	87.5	87.8	87.6	10 A/chicken/Egypt/D7663C/2013
11	2.3	2.3	2.5	2.3	2.3	2.2	3.1	2.7	3.2	3.5		97.2	97.7	96.7	97.3	97.1	97.1	97.0	97.4	97.7	98.0	90.1	95.8	88.9	90.1	82.6	98.0	87.2	88.4	88.2	11 A/chicken/Egypt/F-7297E/2013
12	1.1	2.2	1.7	2.3	2.2	0.9	2.4	1.9	1.5	2.4	2.8		98.3	97.3	98.0	98.8	98.8	98.6	97.5	98.6	98.2	90.5	96.4	89.0	90.6	83.1	98.2	88.4	88.4	88.2	12 A/chicken/Egypt/Q4698A/2012
13	1.4	2.1	1.1	2.2	2.1	1.1	1.1	1.2	2.1	1.9	2.4	1.7		98.8	98.8	98.2	98.2	98.1	97.5	99.2	98.3	90.7	96.6	89.6	90.8	83.0	98.3	88.3	88.6	88.4	13 A/chicken/Egypt/S5018A/2012
14	2.4	3.2	2.2	3.2	3.2	2.1	2.2	2.4	3.2	2.8	3.3	2.8	1.2		97.7	97.2	97.2	97.2	96.5	98.2	97.3	89.8	95.5	88.7	90.1	82.6	97.3	87.7	87.9	87.8	14 A/chicken/Egypt/S5018D/2012
15	1.7	2.5	1.5	2.6	2.5	1.4	1.9	0.2	2.5	2.3	2.8	2.1	1.2	2.3		97.8	98.8	97.7	97.2	98.7	97.8	90.5	96.1	89.4	90.5	83.1	97.8	88.1	88.2	88.1	15 A/chicken/Egypt/S5442C/2012
16	1.2	2.3	1.9	2.3	2.3	1.0	2.5	2.1	0.2	2.6	3.0	1.2	1.8	2.9	2.2		100.0	99.9	97.7	98.4	98.1	90.6	96.3	89.4	90.5	82.5	98.1	89.1	88.4	88.2	16 A/chicken/Egypt/S7018B/2013
17	1.2	2.3	1.9	2.3	2.3	1.0	2.5	2.1	0.2	2.6	3.0	1.2	1.8	2.9	2.2	0.0		99.9	97.7	98.4	98.1	90.6	96.3	89.4	90.5	82.5	98.1	89.1	88.4	88.2	17 A/chicken/Egypt/S7022D/2013
18	1.3	2.4	2.1	2.4	2.4	1.1	2.6	2.3	0.4	2.8	3.1	1.4	1.9	2.9	2.3	0.1	0.1		97.5	98.3	98.0	90.5	96.2	89.2	90.4	82.5	98.0	89.1	88.4	88.2	18 A/chicken/Egypt/S7025C/2013
19	2.1	1.4	2.5	1.4	1.2	1.9	3.2	2.8	2.5	3.3	2.6	2.6	2.5	3.6	2.9	2.4	2.4	2.5		97.7	98.7	90.6	96.4	89.2	90.6	83.0	98.7	87.9	89.2	88.9	19 A/chicken/Egypt/S7404/2013
20	1.2	1.9	0.7	2.0	1.9	0.9	1.5	1.2	1.9	1.6	2.3	1.4	0.8	1.9	1.3	1.6	1.6	1.7	2.3		98.4	90.8	96.6	89.5	90.8	83.1	98.4	88.5	88.7	88.6	20 A/chicken/El
21	1.6	0.9	1.9	1.1	0.9	1.2	2.5	2.1	2.2	2.8	2.1	1.9	1.7	2.8	2.2	1.9	1.9	2.1	1.3	1.6		91.4	97.1	90.0	91.4	83.5	100.0	88.2	89.5	89.4	21 A/quail/Egypt/113413v/2011
22	9.9	9.8	9.9	10.1	9.9	9.7	10.9	10.3	10.5	11.0	10.8	10.4	10.1	11.2	10.4	10.2	10.2	10.4	10.2	9.9	9.3		92.1	94.7	89.7	87.1	91.4	81.5	82.1	81.9	22 A-quail-|HongKong-G1-97-H9N2
23	3.4	3.3	3.4	3.5	3.4	3.1	4.1	4.0	3.9	4.4	4.4	3.7	3.6	4.7	4.1	3.8	3.8	3.9	3.7	3.5	3.0	8.4		90.7	92.4	84.4	97.1	86.7	87.4	87.4	23 A-chicken-Israel-182-2008-H9N2
24	11.7	11.5	11.2	11.8	11.7	11.5	12.2	11.6	12.0	12.6	12.2	12.1	11.4	12.5	11.7	11.7	11.7	11.8	11.8	11.5	10.9	5.5	10.1		88.5	85.6	90.0	80.6	81.1	80.8	24 A/chicken-Israel-375-2007-H9N2
25	10.2	9.9	10.0	10.2	9.9	9.8	10.6	10.4	10.6	10.6	10.8	10.2	10.0	10.8	10.3	10.3	10.3	10.4	10.2	9.9	9.2	11.3	8.1	12.8		82.8	91.4	81.7	82.2	82.0	25 A-chicken-Israel-1163-2011-H9N2
26	15.7	15.0	15.0	15.3	15.3	15.4	16.0	15.4	16.4	15.9	16.1	15.4	15.6	16.0	15.4	16.2	16.2	16.3	15.5	15.4	14.9	10.2	13.7	12.1	15.8		83.5	76.6	77.6	77.5	26 A-duck-|HongKong-y280-97-H9N2
27	1.6	0.9	1.9	1.1	0.9	1.2	2.5	2.1	2.2	2.8	2.1	1.9	1.7	2.8	2.2	1.9	1.9	2.1	1.3	1.6	0.0	9.3	3.0	10.9	9.2	14.9		88.2	89.5	89.4	27 Egypt-H9-1-2014
28	1.6	1.9	2.1	2.6	2.4	1.3	2.8	2.2	1.3	2.9	3.3	1.9	2.0	2.7	2.3	1.1	1.1	1.1	2.5	1.8	2.2	10.4	3.9	11.5	10.1	16.2	2.2		87.9	98.3	28 A/chicken/Egypt/SCU20/2014 (H9N2)
29	1.5	0.8	1.7	0.9	0.8	1.3	2.5	2.0	2.2	2.7	1.9	1.9	1.7	2.4	2.1	1.9	1.9	1.9	1.0	1.5	0.6	9.6	3.0	10.9	9.4	14.8	0.6	2.4		99.2	29 A/chicken/Egypt/SCU8/2014 (H9N2)
30	1.7	0.2	1.7	1.1	1.0	1.4	2.7	2.2	2.4	2.9	2.1	2.1	1.9	2.7	2.3	2.2	2.2	2.2	1.4	1.7	0.8	9.8	3.1	11.3	9.7	14.9	0.8	1.9	0.9		30 A/chicken/Egypt/SCU9/2014 (H9N2)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	

Fig. 2: Amino acid identities of H9N2 with other selected AIV sequences

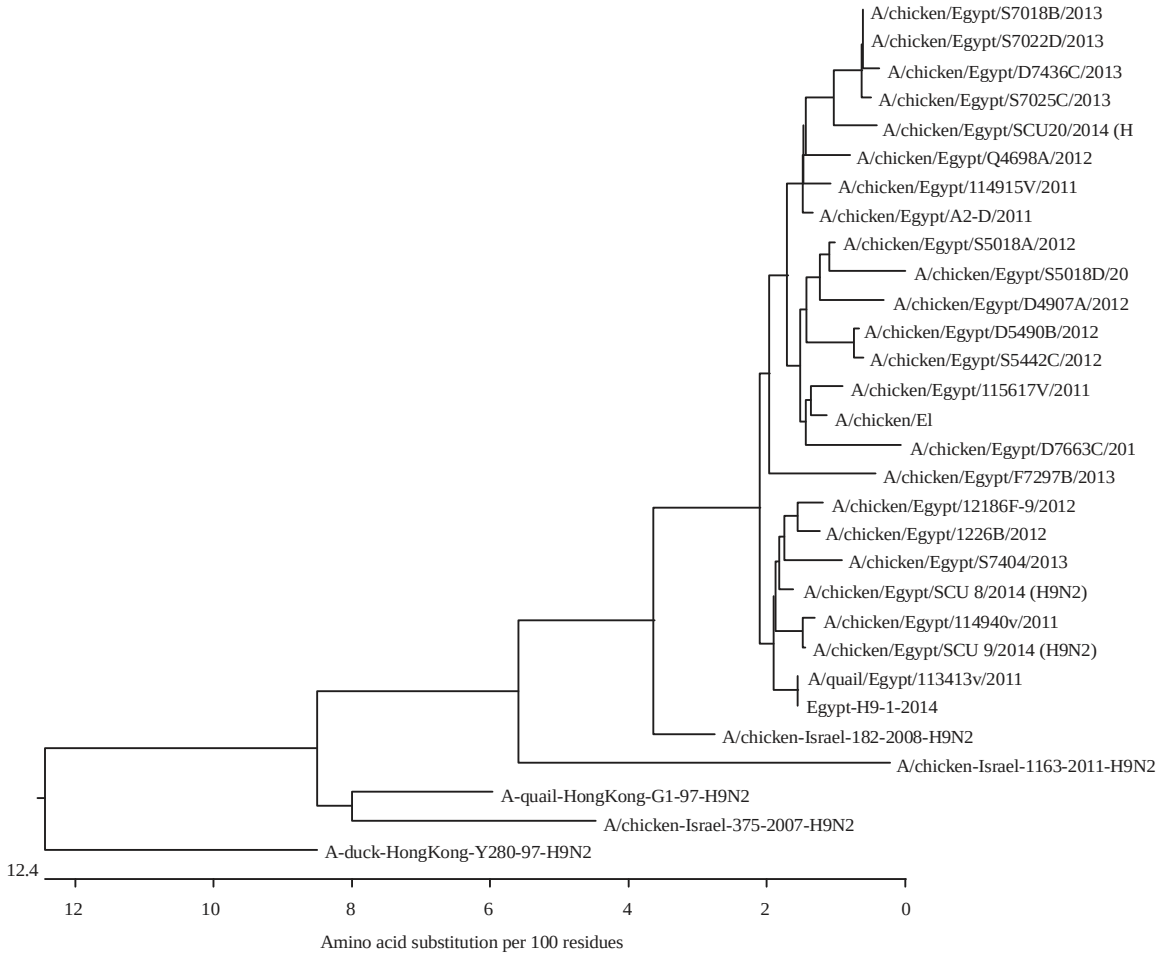

Fig. 3: Phylogenetic tree for the AI (H9) samples based on the amino acids sequence

Table 2: Mortality rate and results of IF and ABP

	Criteria						
Disease	Number of affected broiler farms	Number of affected broilers per flock	Age of affected broilers per flock (days)	Mortality rate (%)	Collected tissue specimen	IF on frozen and/or formalin fixed samples	ABP on formalin fixed samples
AIV	4	4,000	32	20	Broiler trachea	+ve	+ve
					Broiler lungs	+ve	+ve
		6,000	30	30	Broiler spleen	Not applied	Not applied
		3,000	35	10	Broiler kidneys	+ve	+ve
		5,000	28	25	Broiler liver	Not applied	-ve
					Embryonic lung	-ve	-ve
					Embryonic liver	-ve	+ve
					Embryonic kidney	-ve	-ve
					Embryonic intestine	+ve	Not applied

ages of the affected broiler flocks in positively infected farms for H9N2 were variable ranging from 28-35 days. Mortality rate in the positively infected farms ranged from 10-30%. Number, ages and mortality rate of broilers from the positive farms on the day of sampling were summarized in (Table 2). The most frequent gross lesions in the infected birds were turbidity of the thoracic and abdominal air sacs with mild congestion of the trachea and lung. Kidneys were swollen. Other organs did not show obvious gross lesions.

Egg passage: After 72 h PI embryos showed diffuse edema and petechial hemorrhage Fig. 4.

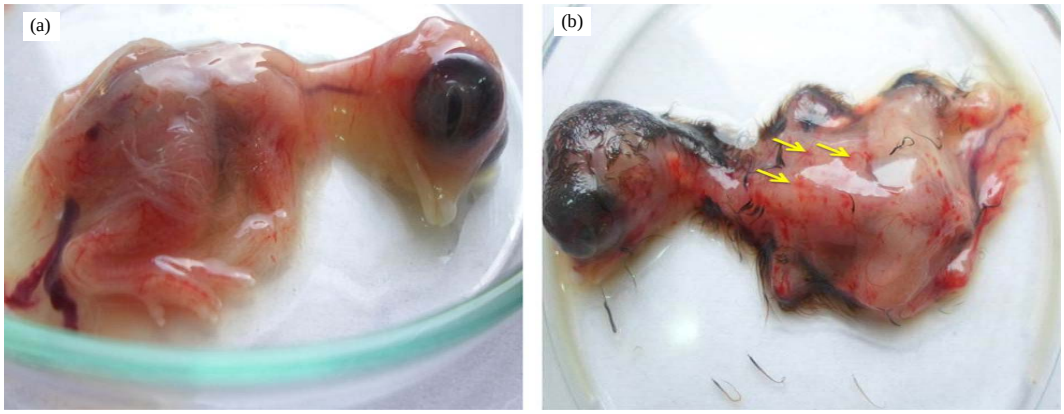

Fig. 4(a-b): Embryos 72 h PI of PCR-positive infected material with H9N2 on allantoic cavity show (a) Diffuse edema and (b) Petechial hemorrhages (yellow arrows)

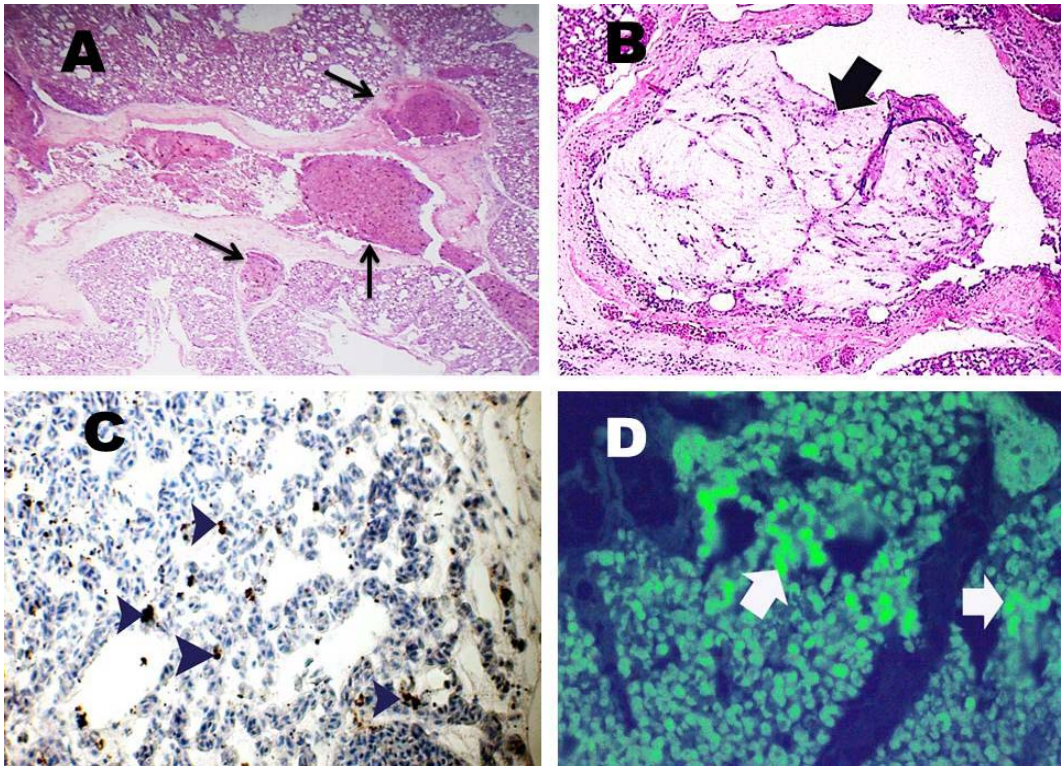

Fig. 5(A-D): Lung of PCR positively infected broiler shows (A) Congested pulmonary blood vessels (arrows) (H and E, 50x), (B) Accumulation of pale bluish mucus and heterophils in lumen of secondary bronchioles (arrow) (H and E, 100x), (C) Positive immunolabelling against H9N2 antigen as dark brown granules inside alveolar epithelium (arrowheads) (ABP counterstained with Mayer's hematoxylin, 200x) and (D) Positive green apple fluorescent dye against H9N2 antigen in frozen lung (white arrows) (indirect IF method, 200x)

Histopathological lesions: Trachea, lungs, spleen, liver and kidneys from PCR-positive broilers were microscopically examined. Trachea revealed necrosis and desquamation of the epithelial lining trachea with infiltration of lymphocytes in mucosa and edema in submucosa Fig. 5A. Positive immunolabelling for H9N2 viral antigen were shown inside the

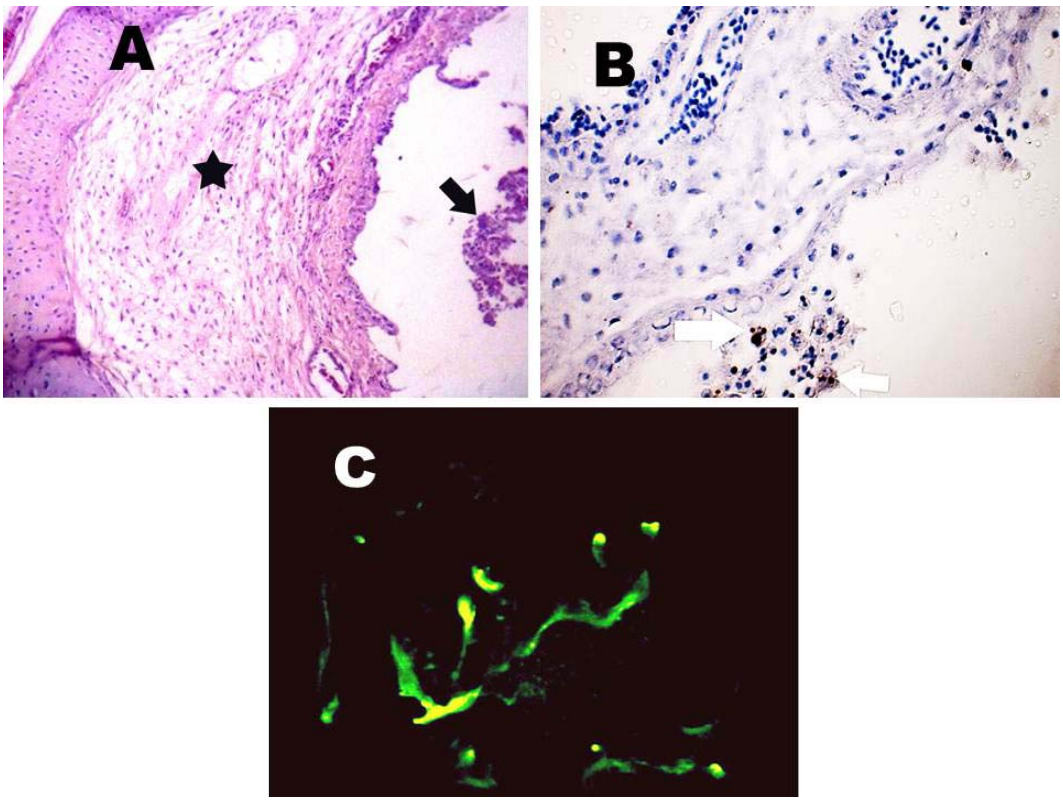

Fig. 6(A-C): Trachea of PCR positively infected broiler with H9N2 shows (A) Necrosis and desquamation of the epithelial lining trachea (arrow) and edema in submucosa (asterisk) (H and E, 100x), (B) Positive immunolabelling against H9N2 antigen as dark brown granules inside desquamted epithelial cells in lumen of trachea (white arrows) (ABP counterstained with Mayer's hematoxylin, 200x) and (C) Positive green apple fluorescent dye against H9N2 antigen in desquamated cells in lumen of frozen trachea (indirect IF method, 200x)

desquamated tracheal epithelium as dark brown granules by ABP in formalin fixed samples (Fig. 5B) and as green apple fluorescent dye in formalin fixed or frozen tracheal samples by IF (Fig. 5C). Lungs showed congestion of pulmonary blood vessels and perivascular hemorrhage (Fig. 6A) with pale bluish mucus and heterophils accumulation in lumen of secondary bronchioles (Fig. 6B). Positive immunolabelling for H9N2 viral antigen were shown inside blood capillaries and alveolar epithelium as dark brown granules by ABP in formalin fixed samples (Fig. 6C) and as a green apple fluorescent dye in formalin fixed or frozen samples by IF (Fig. 6D). Spleen showed necrosis and depletion of lymphocytes from white pulp (Fig. 7A). Liver showed congestion of portal veins with periportal aggregation of leukocytes mainly lymphocytes and macrophages (Fig. 7B). H9N2 viral antigen was not detected in liver tissue by IF. Kidneys revealed interstitial congestion, hemorrhage, vacuolar degeneration in tubular epithelium, degeneration and collapse of glomeruli with peri-glomerular fibrosis (Fig. 8A, B). Positive immunolabelling for H9N2 viral antigen were shown as dark brown granules by ABP or as apple fluorescent dye inside tubular epithelial cells in formalin fixed kidneys.

Lung, liver, kidneys and intestine of inoculated chicken embryos were also examined microscopically. Lung shows congestion in pulmonary blood vessels, interstitial and perivascular edema (Fig. 9A, B). Small intestine showed desquamation of intestinal villi (Fig. 9C). Liver showed congested hepatic sinusoids and degenerative changes in hepatocytes (Fig. 9D). Meanwhile, embryonic kidneys did not show histopathological lesions. Furthermore, mild positive reaction was detected against H9N2 antigen by ABP and IF in formalin fixed embryonic liver (Fig. 10A, B) and by IF in formalin fixed embryonic small intestine (in the muscular layer and serosa) (Fig. 10C, D). Antigen of H9N2 virus was not located in lungs or kidneys of embryos either by ABP or IF.

Results of IF and ABP tests in different tissues from broilers and inoculated chicken embryos were demonstrated in Table 2.

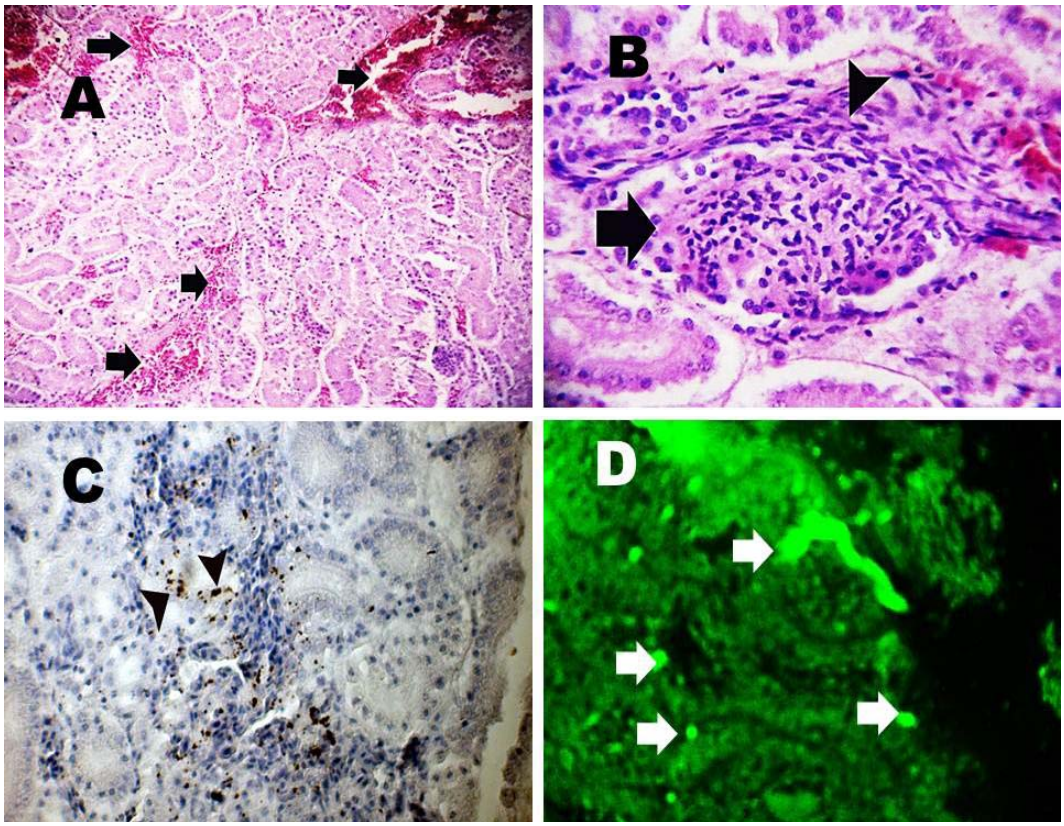

Fig. 7(A-D): Kidney of PCR positively infected broiler with H9N2 shows (A) Interstitial hemorrhage (arrows)(H and E, 100x), (B) Collapsed glomerulus (arrow) with peri-glomerular fibrosis (arrowhead)(H and E, 200x), (C) Positive immunolabelling against H9N2 antigen is seen as dark brown granules inside renal epithelial cells (arrowheads) (ABP counterstained with Mayer's hematoxylin, 200x) and (D) Positive green apple fluorescent dye against H9N2 antigen is seen in renal epithelial cells (white arrows) (indirect IF method, 200x)

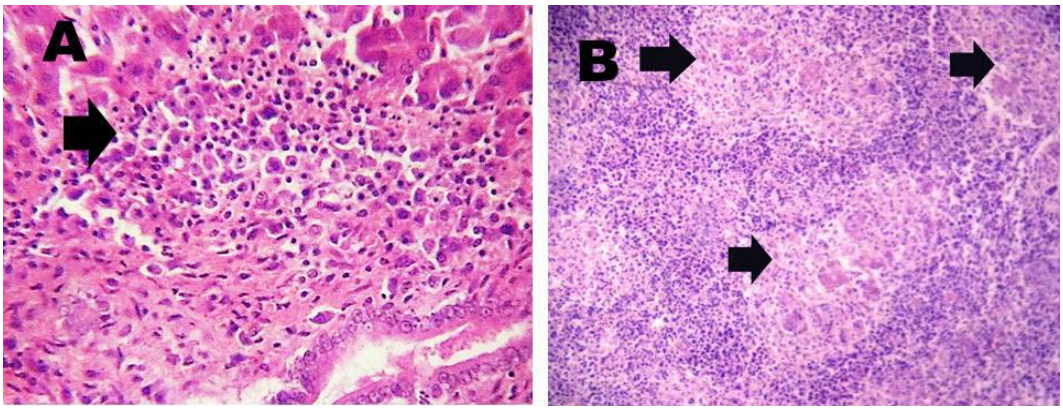

Fig. 8(A-B): (A) Liver of PCR positively infected broiler with H9N2 shows leukocytic cells aggregation (lymphocytes and macrophages) in portal area (arrow) and (B) Spleen of PCR positively infected broiler with H9N2 shows necrosis and depletion of lymphocytes from white pulp (arrows). (H and E, A 200x and B 100x)

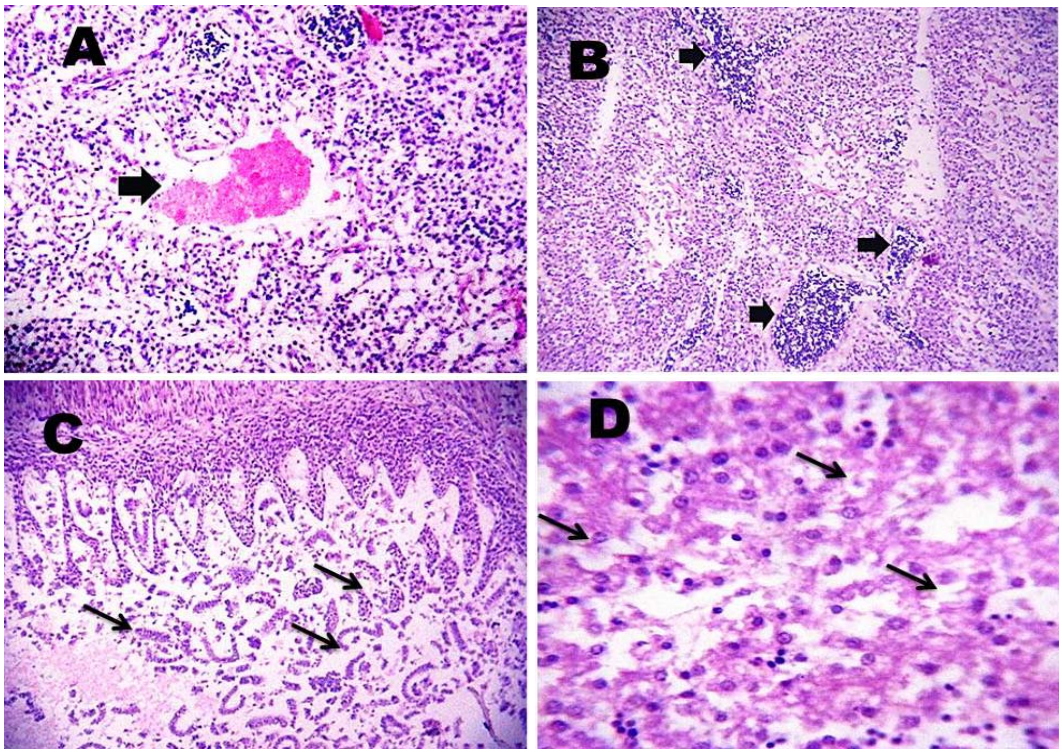

Fig. 9(A-D): Embryonic lung shows (A) Eosinophilic caseated material inside lumen of parabronchus (arrow), (B) Congested pulmonary blood vessels (arrows), (C) Embryonic small intestine shows desquamation of intestinal villi (arrows) and (D) Liver of embryo shows degenerative changes (arrows) (H and E, A and C 100x, B 50x and D 200x)

DISCUSSION

In this study, H9N2 alone was diagnosed by RT-PCR in 4 broiler farms out of 25 during the period from October, 2014 to January, 2015. Phylogenetic analysis of the HA gene showed that the isolates from Egypt were grouped in Quail/Hong Kong/G1/97 lineage-strains similar to the one circulating in the Middle East. The Egyptian H9N2 AIVs were located with Israeli ones in characteristic cluster among G1 lineage. Egyptian H9N2 AIVs were regarded as viruses of low pathogenicity. However, infections with mild or no clinical signs raised the difficulty of exact diagnosis and made the viruses spread rapidly with frequent antigenic variation. Most of the affected birds showed slight depression, low intake of feed and water with mortality rate ranged from 10-30%. No mortality was reported in H9N2 infected birds by Mo *et al.*[20], Banks *et al.*[21], Subtain *et al.*[22]. However, 65 and 30% mortalities were also reported due to H9 subtype by Vasfi Marandi and Bozorgmehrifard[23], Kim *et al.*[24], Jakhesara *et al.*[25]. The clinical signs and lesions found at postmortem examination were almost milder than those produced in naturally infected chickens during H9N2 outbreaks in Iran and Pakistan[4,5,12,26]. The most frequent gross lesions in the infected birds were turbidity of the thoracic and abdominal air sacs, mild congestion in trachea, lungs and liver with swollen kidneys. Other organs did not show obvious gross lesions as previously reported by Hadipour *et al.*[27].

In Egypt, subtype H9N2 viruses were detected in 2011 in poultry from areas where subtype H5N1 viruses circulated[28]. An update was provided by Kayali *et al.*[29] on the changing epizootiology and genetic features of AIV in Egypt and reported co-infection of poultry in Egypt with AIV subtypes H5N1 and H9N2. This huge discrepancy may be due to use of different sub-lineage of H9 virus, route of inoculation, strain and health status of the experimental birds. The host range and adaptation to different host was correlated with amino acids present within and around the HA receptor binding site which can confer different receptor binding specificity[30]. The molecular determinants of pathogenicity and virulence of the HA protein are the HA1/HA2 connecting peptide sequence, specific amino acids (aa) residues at the receptor binding site and the presence or absence of glycosylation sites around the receptor binding site. Based on the deduced amino acid sequences, the HA1-HA2 connecting peptides of the Egyptian

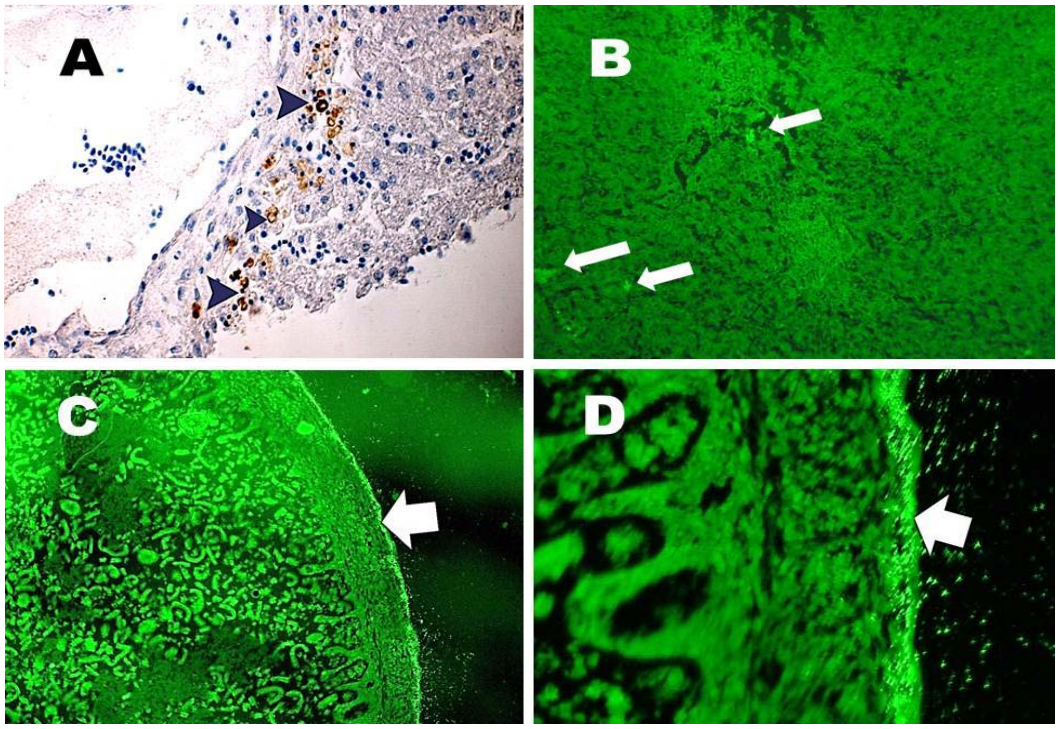

Fig. 10(A-D): Inoculated of embryo shows (A) Positive dark brown immunolabelling of viral antigen in perivascular tissue (arrowheads)(ABP method, 200x), (B) Weak positive green apple fluorescent dye against viral antigen (white arrows) in liver and (C-D) Positive green apple fluorescent dye against viral antigen (white arrow) in small intestine(indirect IF method, B and C 100x, D 200x)

avian H9N2 isolates did not harbour multiple basic amino acids: PARSSR/GL as found for recently isolated H9 viruses in Middle East[14] and Asia[31,32]. This might indicate the LPAI nature of H9N2 strains, although the motif for these viruses was similar to the RX-RYK-R required for the highly pathogenic H5 and H7 subtypes[33, 34]. These genetic findings suggested that our H9N2 viruses may have the potential to acquire basic amino acids in the HA connecting peptide sequence needed to become highly pathogenic through the addition of single basic amino acid at the -4 position. In particular the amino acids glutamine (Q) or leucine (L) at position 226 (H3 numbering, 234H9 numbering) plays a key role in avian and human virus-like receptor specificity, respectively. The presence of leucine (L) at position 226 is associated with mammalian adaptation avian influenza virus. In the present study except for position 226, no additional changes were observed in the HA RBS that could enable avian-human transmission. Similar type of observations were also reported for HA gene of H9N2 viruses prevalent in Israel after detailed molecular analysis and it was found that H9N2 viruses circulating in the Israel are predominantly of G1 lineage and harbors Q226L substitution in HA gene[35,36]. The Q226L substitution seen in RBS of H9N2 avian isolates is important as it confers human virus like receptor specificity and binds more strongly with 2-6-linked sialic acid receptors as seen in human H3N2 viruses[28] in contrast to viruses lacking this substitution which binds more strongly with 2-3 linked sialic acid receptors.

Histopathological changes of trachea, lungs, spleen, liver and kidneys from PCR- positive broilers with H9N2 were agreeing with previous literature[22,37]. Our findings indicated that the target organs for H9N2 in broilers were trachea, lungs and kidneys where the lesions were more pronounced. Previous results showed that the kidneys were the target organ for H9N2 when infection was given by intravenous route Swayne and Slemons[38]. Another study further explained the involvement of lungs with H9N2 virus replication in epithelial tissues of bronchi[22]. On contrary, other findings reported no pulmonary lesions[9,39,40] or mild[41] to non-significant lung lesions[42] when the infection was given by intravenous route. The respiratory involvement may be relied on climatic conditions, subtype of the virus or ability of the virus to replicate in respiratory system after being disseminated throughout the body of the birds via blood. In the current findings, H9N2 antigen was detected

by immunohistochemistry in trachea, lungs and kidneys particularly inside desquamated tracheal epithelium, blood capillaries, alveolar epithelium and inside tubular epithelial cells. Lungs and kidneys were the two main target organs for viral replication and infection persisted in kidneys for longer time[9,10]. H9N2 antigen was previously identified in kidneys and lungs of experimentally infected birds (in the nuclei of pulmonary epithelial cells and within nuclei or cytoplasm of necrotic renal tubular epithelium in kidneys)[22] and in liver of chicken naturally infected with AIV[43-45]. Viral RNA was found by PCR in lung, brain, intestine, peripheral blood mononuclear cells, heart, liver, kidney and spleen from chickens infected with chicken isolated LPAIV H5N2, H7N1, H7N7 or H9N2[44]. Viral antigen of HPAI/H7N3 was detected in areas of necrosis and infiltrating mononuclear cells using immunohistochemistry[45]. Viral antigen was also observed in many tissues, including lymphoid tissues, lung, brain, liver and spleen inside parenchymal cells, cardiomyocytes, Kupffer cells, hepatocytes, microglial cells, neurons, lung cells, kidney tubular epithelial, glomerular cells and feather follicular epithelium. Localization of AIV antigen in tissues may be closely related to each subtype of the virus.

Moreover, lungs, liver and small intestine of inoculated chicken embryos showed different pathological lesions. H9N2 antigen was located in liver and small intestine of inoculated embryos using ABP and IF techniques. It was mentioned that the AIV multiplied in the inner epithelial layer of the amnion, superficial epidermal cells, superficial epithelia of the oropharyngeal cavities, esophagus, nasal and paranasal sinuses[46]. Kidneys were free of virus antigen, although the virus multiplied to high titers in primary tissue cultures derived from embryonic kidneys. It was not clear why three germinal layers of the allantoic membrane in embryonated eggs did not function as a tight barrier against AIV[47]. In addition, primary chicken embryo hepatocytes were used for virus propagation, detection and subsequent vaccine production[48,49]. Thus, site of replication of the H9N2 virus in tissues differed in broilers from that in embryos.

CONCLUSION

- AIV subtype H9N2 circulating in broiler farms in Dakahlia province during the period from October, 2014 to January, 2015 was grouped in Quail/Hong Kong/G1/97 lineage- strains similar to the one circulating in the Middle East
- Site of replication of the H9N2 virus in tissues differed in broilers from that in embryos as it showed affinity for lungs, trachea and kidneys in broiler chickens, liver and intestine in embryos

SIGNIFICANCE STATEMENT

This study discovered that H9N2 circulating in Dakahlia province, Egypt during the period from October, 2014 to January, 2015 was low pathogenic in nature, however, the use of biosecurity measures by poultry farmers is recommended to avoid outbreaks. This study will help the researcher to know site of replication of the H9N2 virus in broilers and embryos. Thus a new theory on tissue virus tropism may be arrived at

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