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Comparative molecular characterization, pathogenicity and seroprevalence of avian influenza virus H9N2 in commercial and backyard poultry flocks

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ABSTRACT

This study was conducted to perform the comparative molecular characterization of avian influenza virus (AIV) H9N2, pathogenicity and seroprevalence in commercial and backyard poultry flocks. Fifty commercial poultry flocks were investigated between 2012 and 2015. Eighteen flocks (36%) out of 50 were positive HA. Seven (38.9%) out of 18 were positive by chromatographic strip test for AI common antigen. By Real-time RT-PCR, only two flocks were positive H9. The molecular characterization of two different AI-H9N2 viruses, one isolated from a broiler flock (A/chicken/Egypt/Mansoura-18/2013) and the other from a layer flock (A/chicken/Egypt/Mansoura-36/2015) was conducted on HA gene. Moreover, a higher seroprevalence, using the broiler strain as a known antigen, was shown in backyard chicken flocks 15/26 (57.7%) than duck flocks 9/74 (12.2%). Interestingly, the pathogenicity index (PI) of the H9N2 broiler strain in inoculated experimental chickens ranged from 1.2 (oculonasal route) to 1.9 (Intravenous route). The PI indicated a highly pathogenic effect, with high mortality (up to 100%) in the inoculated chickens correlated with the high mortality (80%) in the flock where the virus was isolated. The firstly recorded clinical signs, including cyanosis in the combs and wattles and subcutaneous haemorrhages in the leg shanks and lesions, as well as histopathology and immunohistochemistry, revealed a systemic infection of the high pathogenicity with the H9N2 virus. Conversely, the H9N2 layer strain showed a low pathogenicity. In conclusion, as a first report, the molecular analysis and pathogenicity of the tested strains confirmed the presence of a high pathogenicity AIV-H9N2 with systemic infections.

1. Introduction

The avian influenza virus H9N2 (AIV-H9N2) is a low pathogenic avian influenza (LPAI) caused by type A influenza viruses that cause natural infection in birds belongs to *Orthomyxoviridae* family [1]. In poultry infected with H9N2, clinical signs generally range from 0% to 97% mortality in severe cases with great economic losses [1]. The signs of H9N2 exacerbation were explained [2] that cleavage occurs by proteases that enhance the replication of the virus in chickens. So the result in an exaggeration of the H9N2 virus infection that causes systemic infections and high rates of mortality. The reason for increasing the pathogenicity of H9 is the stimulation of the host cell to secrete more proteases [3].

The signs of viral disease in pathogenicity test vary depending on the dose and concentration of the virus; and this is true even with LPAI-H9 [4]. The clinical signs of experimental infection with pneumotropic H9 avian influenza strains were more exacerbated through intranasal route [5]. The virus can spread to different body organs under certain conditions, such as stress, which increases its cleavage in different organs which have epithelium with trypsin-like protease and receptors; therefore, the mortality may be increased up to 97%, especially in young birds [1].

H9N2 is endemic in backyard birds, which play an important role in transmitting the virus to other birds in the commercial sector and in live bird markets [6]. The backyard poultry flocks showed that the rearing of different species, ages, and breeds at the same place gave an idea about the smooth transmission of any disease between birds and the absence of any biosecurity measures. The lack of immunization against any disease, including H9-AIV, served us in the interpretation of the seroprevalence [7].

In Egypt and the Middle East, no thorough studies have been carried out on evolving H9N2 virus using molecular analysis of viruses with an

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increase of pathogenicity indices and mortality rates. To the best of our knowledge, there are few published articles on the seroprevalence of H9N2 on backyard flocks of chickens and ducks in Egypt [6,8]. This study aimed to: (I): Collect suspected samples of H9N2 from the infected commercial flocks of different breeds of chickens (broiler and layer). (II): Determine the seroprevalence of H9N2 infection in the backyard poultry flocks using the prepared isolated antigen. (III): Analyse the molecular characteristics of the isolated H9N2 based on sequencing and phylogenetic analysis. (IV): Assess the effect of H9N2 infection on chicken health and measure its pathogenicity, including detection of antibody titers, using the HI test, histopathology and immunohistochemical staining.

2. Materials and methods

2.1. Sampling procedures

Fifty commercial poultry flocks (broilers, layers, and breeders) with respiratory and/or mortality rates were investigated between 2012 and 2015 in Dakahlia Governorate, Egypt. The molecular analysis of two different AI-H9N2 viruses of different chicken flocks with different ages and localities, one isolated from a broiler flock in December 2013 and the other from a layer flock in April 2015 was conducted. Clinical signs and lesions of birds were recorded. Samples were collected from the tissues (trachea and lung) of freshly dead birds, and tracheal and cloacal swabs were collected from live or freshly dead birds and were prepared as described by OIE (World Organisation for Animal Health) [9]. For determination of the seroprevalence of backyard poultry flocks, blood samples (n = 10) were collected from 100 house-hold backyard poultry flocks (26 chicken flocks and 74 duck flocks) of different ages and from different localities between 2012 to 2015 in Dakahlia Governorate, Egypt. Numbers of birds in each small size flock were ranged from 20 to 70 birds. Blood samples were collected from the wing vein in the chickens or from the saphenous vein in the ducks, and then the serum samples were separated and stored at −20 °C until used. Poultry flocks used for blood sampling showed respiratory signs or even seemed apparently healthy, especially ducks. Isolate 18B (A/chicken/Egypt/Mansoura-18/2013) of this study was used as a known H9-antigen for measuring HI-antibody titers of unknown serum [10] to establish a correlation between the H9N2 virus monitoring in commercial & seroprevalence in backyard poultry flocks.

2.2. Isolation and identification of the prepared samples

Tissue samples from each flock were pooled and homogenized and then prepared as previously described (9). HA test was performed as previously indicated [10]. The positive HA samples were screened using a rapid chromatographic strip test (ANIGEN® Animal Genetics, Inc. Korea).

2.3. Real- time RT-PCR (rRT-PCR) and conventional RT-PCR

The viral RNA was extracted using the QIAamp viral RNA Mini kit (GmbH, Qiagen, Germany) as described in the manufacturer's instructions. PCR amplifications were performed in a final volume of 25 µl containing 7 µl of RNA template, 12.5 µl of 2X QuantiTect Probe RT-PCR Master Mix, 4.125 µl PCR grade water, 0.5 µl of each primer (50 pmol conc.) and 0.125 µl of probe (30 pmol conc.), and 0.25 µl of QuantiTect RT Mix. Reverse transcription was done at 50 °C for 30 min. followed by primary denaturation at 94 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing, and extension at 60 °C for 45 s. The reaction was done in Stratagene MX3005 P real-time PCR machine. A set of primers and probes of the H9, H7 and H5-AIV genotypes in Real-time RT-PCR (Metabion, Germany) were used (Table 1) and the Ct (cycle threshold) values were determined. Moreover, different primers were used in the conventional RT-PCR to ensure that the isolates were purified viruses and free from contamination by H5 AIV and NDV [11–17] (Table 1). The isolates were examined using two pairs of primers for the H9 and the positive controls were used in the Real-time RT-PCR and RT-PCR tests.

2.4. Sequencing and phylogenetic analysis

QIA Quick Gel Extraction Kit (Qiagen, Inc., Valencia, CA, USA) was used and a purified RT-PCR product was sequenced on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA) using the BigDye™ Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems, Foster City, CA). The primers (Metabion, Germany) were used for sequencing of the HA cleavage site. The obtained sequence data were analyzed using ClustalW (http://www.ebi.ac.uk/clustalw/) and then the alignment output file was used for performing the phylogenetic Neighbor-Joining analysis. Sequence comparisons were conducted with the Megalign program (DNASTAR, Lasergene®). A BLAST analysis (Basic Local Alignment Search Tool) was initially performed to establish sequence identity to Genbank accessions. Sequence identity was compared with several isolates on the Genbank from Egypt and other countries.

2.5. GenBank accession numbers

The nucleotide sequences of the HA1 gene and the amino acids of the 2 isolates from this study were deposited in the GenBank NCBI. The accession numbers of both isolates are available as KX663331 and KX663332.

2.6. Pathogenicity test

Pathogenicity test was done according to OIE [9]. Pathogenicity test of the titrated two isolates (36 L and 18B) with EID_{50} [18] was assessed by intravenous (I/V) inoculation with positive and negative controls. The inoculum of each isolate was tested for bacterial contamination by culturing on different media [19]. Positive control H9N2 strain (A/chicken/Egypt/1/2014) was kindly provided [20]. Fresh infective allantoic fluid with an HA titer > 1/16 had been diluted to 1/10 in sterile saline, then 0.1 ml of the diluted virus was injected I/V into ten, 42-day-old white Hy-Line chickens. Moreover, oculonasal (O/N) route of pathogenicity index was used with different concentrations of the virus (1/10, 1/100, 1/500, and 1/1000) and the inoculated birds (n = 10, each dilution) were observed daily for 10 days and scored as previously described [9]. Serum samples were collected pre-challenge and one-week post-challenge for detection of the antibody titers against H9N2, H5N2 and NDV known antigens using HI test [10]. The H9N2 antigen (A/Chicken/Egypt/11490v/NLQP/2011) and the H5N2 antigen (A/Chicken/Egypt/Q1995D/2010) are standard diagnostic avian influenza commercial antigens that were kindly provided by Middle East for veterinary vaccines (ME VAC), Egypt and also La Sota (Intervet, Egypt) was used as a NDV known-antigen.

The bird experiment was reared under 'Guide for the Care and Use of Laboratory Animals' that was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University. Day-old White-Hy line chicks (n = 110) of the same hatching batch were obtained from commercial poultry farm (Dakahlia, Egypt) without a history of H9N2 infection and were divided into 11 groups and subgroups (11 × 10). All birds were reared in separate cages, kept in the strictly isolated room with biosafety conditions and were provided with a commercial starter balanced ration free from any medications. The feed and water were offered *ad-libitum* to all the birds throughout the experimental period (52 days). Birds were confirmed to be free from H9N2 by virus isolation in embryonated eggs and haemagglutination inhibition (HI) test [21].

Table 1
Sequences of primers and probes used in Real time RT-PCR (rRT-PCR) and conventional RT-PCR.

Test	Target gene	Primer or probe sequence (5'-3')	Amplicon length(bp)	References
rRT-PCR	**H9**	**H9F: 5'-GGAAGAATTAATTATTATTGGTCGGTAC-3'**	–	[11]
		H9R:5'-GCCACCTTTTTCAGTCTGACATT-3'	–	
		H9 Probe: [CY5] AACCAGGCCAGACATTGCGAGTAAGATCC [BHQ]	–	
	H5	**H5F:5-ACATATGACTAC CCACARTATTCAG-3**	–	[12]
		H5R:5-AGACCAGCT AYCATGATTGC-3	–	
		H5PRO: [FAM] TCWACAGTGGCGAGTTCCCTAGCA [TAMRA]	–	
	H7	**LH6H7: GGC CAG TAT TAG AAA CAA CAC CTA TGA**	–	[13]
		RH4H7: GCC CCG AAG CTA AAC CAA AGT AT		
		H7 pro11:6-HEX-5′-CGCTGCTTAGTTTGACTGGGTCA ATC T- BHQ-3′		
Conventional RT-PCR	**H9**	**H9F:**5-GAATCCAGATCTTTCCAGAC-3	**383 bp**	[14]
		H9R:5-CCATACCATGGGGCAATTAG-3		
	H9	**H9F:**5-ATCGGCTGTTAATGGAATGTGTT-3	**221 bp**	[15]
		H9R:5-TGGGCCTCTTGAATAGGGTAA-3		
	H5	**H5F:**5-ACAAAGCTCTATCAAAACCCAAC-3	**499 bp**	[15]
		H5R:5-TACCCATACCAACCATCTACCAT-3		
	H5	**H5F:**5-GCCATTCCACAACATACACCC-3	**219 bp**	[16]
		H5R:5-CTCCCCTGCTCATTGCTATG-3		
	ND	**NDF:5-**GCAGCTGCAGGGATTGTGGT-3	**356 bp**	[17]
		NDR:5-TCTTTGAGCAGGAGGATGTTG-3		

2.7. Histopathology and immunohistochemical staining

Tissue samples for histopathology had been collected from different organs (lung, trachea, pancreas, kidney, proventriculus, intestine, spleen, and bursa of Fabricius) of morbid or freshly dead challenged birds (n = 4) at 5–10 days post-infection from all groups All samples were placed in 10% neutral buffered formalin then sectioned and stained with H&E stain as previously described [22]. Moreover, tissue samples for immunohistochemical staining had been collected from different organs (lung, trachea, liver, spleen, kidney and intestine) of morbid or freshly dead birds (n = 4) at 5–10 days post-infection from all groups. Unstained paraffin-embedded sections were prepared and immunohistochemically stained by using the polyclonal antibody against nucleoprotein of type A influenza virus subtype H9N2 [23].

2.8. Statistical analysis

The student *t*-test was used to compare the variables and significance between the two groups of HI titers pre- and post-challenge. The data were performed with statistical software [24].

3. Results

3.1. Clinical findings, lesions and the seroprevalence

Fifty commercial poultry flocks (broilers, layers, and breeders) with respiratory and/or mortality rates were investigated. Eighteen flocks out of 50 (36%) were haemagglutination (HA) positive. Seven out of 18 (38.9%) were positive by chromatographic strip test for AI common antigen. By Real-time RT-PCR, only two flocks were H9 positive. The two flocks were examined (one broiler and one layer chicken flock). The first examined flock (broiler flock 18B) showed respiratory signs (coughing, tracheal rales, sneezing, difficult breathing, conjunctivitis, nasal and ocular discharges), sudden death, and white diarrhoea, with 80% mortality, while post-mortem lesions revealed an enlarged spleen, congested trachea, pneumonia, and fibrinous pericarditis. Meanwhile, the second examined flock (layer flock 36 L) showed a 50% drop in egg production, respiratory signs, 10% mortality, congested trachea, pneumonic lung, congested duodenum, and petechial haemorrhage in the pectoral muscle (Table 2). The seroprevalence of H9 positive backyard poultry flocks using the broiler strain as a known antigen was performed. The descriptive data for H9 positive backyard chicken flocks (by HI titers) were indicated in (Table 3). The clinical signs of backyard chickens showed respiratory manifestations, such as sneezing, coughing, nasal discharge, gasping, rales, respiratory distress, and a swollen head. Moreover, whitish diarrhea, depression, a decrease of food consumption and mortality ranged from 2 to 5 birds daily for each flock. Meanwhile, backyard duck flocks were apparently healthy and the post-mortem examination was not done. Regarding seroprevalence, 15 flocks (57.7%) were positive out of 26 chicken flocks examined and the mean HI titers ranged from 1.75 to 7.5 (log2). Meanwhile, 9 flocks (12.2%) were positive out of 74 duck flocks examined and the mean HI titers were ranged from 0.66 to 4.33 (log2) as shown in (Table 4).

3.2. Avian influenza virus (AIV) H9 isolation and identification

The suspected samples (tracheal swabs and lung tissues) of the two flocks with H9N2 revealed the death of embryos in specific pathogen–free eggs at 2–5 days post-inoculation during H9N2 isolation. Congestion of the embryos and petechial haemorrhage were observed. The real-time RT-PCR results showed two positive isolates (18B and 36 L) for H9-AIV, with Ct values of 22.15 and 19.57, respectively. Both isolates were also tested for AI-H5, H7 and ND viruses, but the results were negative. The two isolates were titrated, giving $10^{8.5}$ and 10^{8} EID_{50} for 18B and 36 L, respectively (Table 2). The two isolates were also subjected to the pathogenicity test.

3.3. Conventional RT- PCR

Positive results were shown for the H9 virus, while negative findings emerged for the H5, and ND viruses, confirming that the isolates (18B and 36 L) were purified H9N2 viruses and free from contamination with these viruses (Table 2).

3.4. Phylogenetic and Sequence analysis of the HA gene

A partial *HA* (*hemagglutinin*) gene of the two H9 viruses (18B and 36 L) isolated were sequenced (Table 2). Both sequences were submitted to the GenBank database (NCBI). Both isolates had the cleavage site sequence PARSSRGLF, which is a feature of low-pathogenic AIV. Phylogenetic tree analysis showed that both isolates were clustered with other Egyptian isolates and grouped into the Quail/Hong Kong/

Table 2
Descriptive data of the examined broiler and layer flocks infected with H9 avian influenza virus.

Items		Broiler flock (18B isolate)	Layer flock (36 L isolate)
History		Manzala city, 12,000 broilers, 27 days old, and 2/12/2013 (date of sample collection)	Mansoura city, 10,000 laying hens, 420 days old, and 23/4/2015 (date of sample collection)
Clinical signs		Respiratory signs, sudden death, whitish diarrhea with 80% mortality	50% drop in egg production, and respiratory signs, with 10% mortality
Postmortem lesions		Enlarged spleen, congested trachea, pneumonic lung and fibrinous pericarditis.	Congested trachea, pneumonic lung, congested duodenum, and petechial hemorrhage on pectoral muscle.
Plate HA-test		+	+
Rapid chromatographic (common antigen) test		+	+
Real-time RT-PCR (CT value)		**22.15**	**19.57**
Titration (EID_{50})		$10^{8.5}$	10^{8}
RT-PCR (primers)	**H9 AIV**	+	+
	H5 AIV	–	–
	ND virus	–	–
Strain name on Gene bank (accession no.)		**A/chicken/Egypt/Mansora-18/2013 (KX663331)**	**A/chicken/Egypt/Mansora-36/2015 (KX663332)**
nucleotides sequenced		**621 nucleotides**	**684 nucleotides**
Cleavagesite		**PARSSR/GLF**	**PARSSR/GLF**
Identity% withparentstrain from quail		**98%**	**99%**
Identity% withthe firstchickenisolateinEgypt		**99%**	**98%**
Identity%withGallimune-208®H9N2 strain vaccine		**91%**	**92%**
Amino acid substitutions in HA sequenced genecompared to H9 strain G1		**10 amino acid substitutions (Y10 F, G17E, K28 N, S29 N,T59D, V64I, R65 G, K67 T, V73I, and I129 V)**	**10 amino acid substitutions (Y10 F, G17E, K28 N, T59D, V64I, R65 G, V73I, A103S, I129 V, and D141 G)**

Table 3
Descriptive data for positive HI-titers of poultry backyard flocks for H9-AIV seroprevalence.

Chickens' backyard flocks					Ducks' backyard flocks				
Flock no.	No. of birds	Age of birds (week)	Date of collection	Mean HI titers (Log2) n = 10	Flock no.	No. of birds	Age of birds (week)	Date of collection	Mean HI titers (Log2) n = 10
1	50	4	11/2012	3.25	**1***	45	16	11/2012	3.33
2*	50	3	11/2013	7.50	**2**	20	12	11/2012	1.66
3	50	12	12/2013	5.50	**3**	20	19	12/2013	2.60
4*	50	8	1/2014	4.75	**4**	35	9	12/2013	0.66
5*	30	28	1/2014	4.00	**5***	25	4	1/2014	3.00
6	50	4	1/2014	3.75	**6***	32	13	1/2014	4.33
7	40	11	2/2014	3.25	**7**	27	6	2/2014	4.33
8	40	6	3/2014	5.00	**8**	30	20	1/2015	2.33
9	70	7	11/2015	7.25	**9***	22	24	1/2015	1.33
10	40	18	11/2015	2.25					
11	40	15	11/2015	1.75					
12	40	13	12/2015	4.00					
13	50	8	12/2015	2.25					
14*	20	20	12/2015	1.75					
15	50	6	1/2015	2.75					

* mixed flock (duck & chicken).

G1/97 lineage. They are comparable to the viruses circulating in the Middle East like in Fig. 1. Phylogenetic analysis of the H9 subtype of the two isolates compared with the sequences from the 55 H9 subtype viruses available in the GenBank database. There was some degree of variation at the level of the nucleotide sequence with the Quail/Hong Kong/G1/97 strain.

The two isolates shared 98% similarity with each other. In our study, the HA1 genes in the isolates were compared using Gallimune-208® (KF800947.1-A/chicken/Iran/AV1221/1998), the vaccine strain currently in use in Egypt, giving 91–92% identity. When the strains were compared with the first Egyptian quail isolate (JN828570.1-A/Quail/Egypt/113413v/2011), the identity percent was 98–99%; when compared with the first Egyptian chicken isolate (JQ611704. A/chicken/Egypt/BSU-CU/2011), it was also found to be 98–99%, as shown in Table 2.

The 36 L isolate (A/Chicken/Egypt/Mansoura-36/2015) showed

Table 4
Mean HI-antibody titers (Log 2) and seroprevalence% in the examined poultry backyard flocks for H9-AIV.

Type of birds	Total no. of flocks	No. of positive HI flocks	Mean HI titers (Log2) (n = 5)	Seroprevalence %
Chickens	**26**	**15**	**1.75 to 7.50**	**57.7%**
Ducks	**74**	**9**	**0.66 to 4.33**	**12.2%**

Prevalence% = (no. of cases/population size) X100.

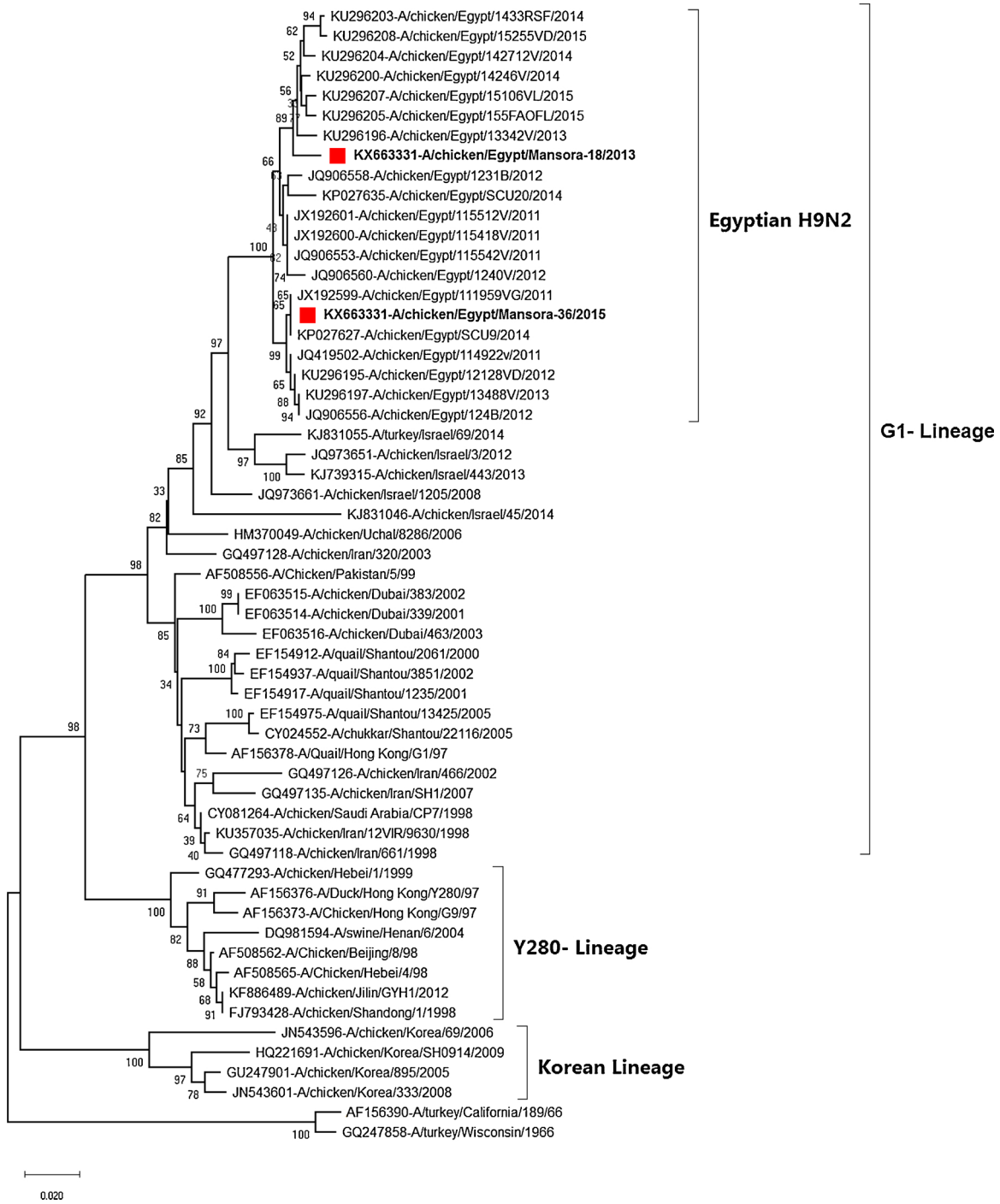

Fig. 1. A phylogenetic tree relationship of the Egyptian isolates 18B (2013) and 36 L (2015) among influenza A virus isolates of H9 subtype viruses available in the Genbank database. A sequence aligned by Clustal W method and the tree was built by using MEGA5 software. A genetic branch distance which indicated below the tree is proportional to the nucleotide. Isolates in this study are marked with solid red rectangular.

substituted amino acids at 10 sites (Y10 F, G17E, K28 N, T59D, V64I, R65 G, V73I, A103S, I129 V, and D141 G). Meanwhile, the isolate 18B, KX663331-A/Chicken/Egypt/Mansoura-18/2013 showed substituted amino acids at also 10 sites (Y10 F, G17E, K28 N, S29 N, T59D, V64I, R65 G, K67 T, V73I, and I129 V). The two studied isolates shared other Egyptian isolates in 8 amino acid substitutions (Y10 F, G17E, K28 N, T59D, V64I, R65 G, V73I, and I129 V) (Table 5).

3.5. Pathogenicity test

A pathogenicity test was carried out for the two H9 isolates (36 L and 18B) and the results are shown in Table 6. Interestingly, the pathogenicity index (PI) of H9N2 broiler strain in the inoculated experimental chicks ranged from 1.2 (O/N route) with 10^6 EID_{50} to 1.9 (IV route) with $10^{7.5}$ EID_{50} while, H9N2 viruses do not commonly show such high pathogenicity index. This PI indicated a highly pathogenic effect, with high mortality (up to 100%) in the inoculated chicks.

In this test, we observed that the clinical signs started to appear at a virus concentration of 1/1,000, with O/N route with $10^{5.5}$ EID_{50}; these signs included whitish diarrhoea, cyanosis in the comb and wattle and subcutaneous haemorrhage in the leg shanks, as a first record. In some cases, sudden death occurred without any clinical signs. The post-

Table 5
Comparison of amino acids substitution among the two studied isolates (18B and 36 L), other Egyptian isolates and the Quail/Hong Kong/G1/97 strain.

Other Egyptian isolates	18B isolate	36L isolate	Quail/Hong Kong/ G1/97 strain	Site of amino acid
F	**F**	**F**	**Y**	**10**
E	**E**	**E**	**G**	**17**
N	**N**	**N**	**K**	**28**
S	**N**	**S**	**S**	**29**
D	**D**	**D**	**T**	**59**
I	**I**	**I**	**V**	**64**
G	**G**	**G**	**R**	**65**
K	**T**	**K**	**K**	**67**
I	**I**	**I**	**V**	**73**
A	**A**	**S**	**A**	**103**
V	**V**	**V**	**I**	**129**
D	**D**	**G**	**D**	**141**
8	**10**	**10**	**Total**	

mortem lesions were petechial haemorrhage on the proventriculus, congested trachea and lung. The mortality pattern was changed by changing the dose of the virus. The pathogenicity index (PI) was 0.8 for 10^6 EID_{50} with a small dose of the virus (0.2 ml) (Table 6).

Clinical signs, lesions, pathogenicity index and mortality rate of other infected groups (positive control and 36 L) indicated the clinical features of low pathogenicity as shown in Table 6. Clinical signs included sneezing, rales, nasal/ocular discharge, conjunctivitis, and head swelling, while congested trachea and lung and swollen kidney were shown in lesions.

Post-challenge, the H9 virus was re-isolated from different organs (trachea and lung) and swabs (tracheal and cloacal) and gave positive AIV when tested by HA and a chromatographic strip test, and H9 was reconfirmed by real-time RT-PCR in all infected groups.

No maternal HI-antibody titers of AIV-H9N2 were detected in the experimental chicks at the 1st day or 25th day of age. The HI antibody titres for H9 virus significantly ($P \leq 0.05$) increased, and the seroconversion was changed from 0.0 HI titers pre-challenge to 6.8 and 5.0 post-challenge for 18B and 36 L isolates respectively, while the HI titers for H5-AIV and NDV remained the same (0.0; Table 7). It is worth to mention that this experiment was repeated twice.

3.6. Histopathology and immunohistochemical staining

Histopathological changes in infected tissues of different organs with H9N2 (18B isolate) were reported and revealed a systemic infection (Fig. S1 & S2). Immunohistochemistry of lung, trachea, liver, spleen, kidney and intestine of positively infected chickens with H9N2 (18B isolate) showed also positive immunolabelling of H9N2 antigen by the immunoperoxidase method and revealed a systemic infection (Fig. S3).

4. Discussion

This study was conducted on the molecular analysis of avian influenza H9N2 viruses, with pathogenicity and seroprevalence in backyard chicken and duck flocks. In our study, two field H9N2 isolates from commercial poultry flocks were designated as a broiler strain (KX663331-(A/chicken/Egypt/Mansoura-18/2013) and a layer strain (KX663332- A/chicken/Egypt/Mansoura-36/2015) and were analyzed. Our observations in the backyard poultry flocks showed that the rearing of different species at the same place and the lack of immunization against diseases, including H9-AIV, served us in the interpretation of HI results of seroprevalence [7]. We supposed that any HI titer was considered as a positive result for infection due to the absence of vaccination in the history of backyard birds. Regarding our results on H9N2 seroprevalence, we found that chickens and ducks in the area of study were exposed to infection with H9N2 and the chickens showed more susceptibility than ducks. Therefore, the vaccination of the commercial and backyard chickens or ducks against avian influenza virus H9N2 should be necessarily conducted. This is to evade the infection and spread of the emergent and virulent strains of this virus. To the best of our knowledge, there are few published articles about the serosurvey of H9-AIV in the backyards, at least in Egypt [6,8].

Miniature seroprevalence of H9-AIV in backyard chickens in our study was 57.7% and HI titer ranged between 1.75 to 7.5 log2,

Table 6
Pathogenicity index of H9 avian influenza isolate (18B) with $10^{8.5}$ EID_{50} and (36 L) with 10^8EID_{50} tested with positive and negative control in white Hy-Line chicks (42-day-old) with different concentrations and routes.

Groups		Conc.	EID_{50}	Route	Dose	No. of birds	Mortality post-infection										Total (%)	Pathogenicity Index (PI)
							1	2	3	4	5	6	7	8	9	10		
G1	**Negative control**	–	–	–	–	**10**	–	–	–	–	–	–	–	–	–	–	**0/10 (0)**	**0.0**
G2A	**positive control**	1/10	$10^{7.5}$	**I/V**	**0.1**	**10**	–	–	–	–	–	–	**1**	**1**	–	–	**2/10 (20)**	**0.2**
G2B	**positive control**	1/10	$10^{7.5}$	**O/N**	**0.1**	**10**	–	–	–	–	–	–		–	–	–	**0/10 (0)**	**0.01**
H9 avian influenza isolate (18B) with $10^{8.5}$ EID_{50}																		
G3A	**18B isolate**	1/10	$10^{7.5}$	**I/V**	0.1	**10**	–	**9**	**1**	–	–	–	–	–	–	–	**10/10 (100)**	**1.9**
G3B	**18B isolate**	1/10	$10^{7.5}$	**O/N**	0.2	**10**	–	**8**	**2**	–	–	–	–	–	–	–	**10/10 (100)**	**1.8**
G3C	**18B isolate**	1/100	$10^{6.5}$	**O/N**	0.2	**10**	–	**2**	–	**1**	–	**1**	–	–	–	–	**4/10 (40)**	**1.6**
G3D	**18B isolate**	1/500	10^6	**O/N**	0.2	**10**	–	–	–	–	–	–	–	**8**	–	–	**8/10 (80)**	**0.8**
G3E	**18B isolate**	1/500	10^6	**O/N**	0.4	10	–	**4**	**3**	**0**	**2**	**1**	–	–	–	–	**10/10 (100)**	**1.2**
G3F	**18B isolate**	1/1000	$10^{5.5}$	**O/N**	0.2	**10**	**1**	**1**	**0**	**2**	**1**	–	–	–	–	–	**5/10 (50)**	**1.1**
H9 avian influenza isolate (36 L) with 10^8EID_{50}																		
G4A	**36 L isolate**	1/1000	10^5	**O/N**	0.2	**10**	–	–	–	–	–	–	–	–	–	–	**0/10 (0)**	**0.0**
G4B	**36 L isolate**	1/100	10^6	**I/V**	0.1	**10**	–	–	–	–	–	–	–	**2**	–	–	**(2/10) (20)**	**0.2**

I/V = intravenous O/N = oculonasal.

Table 7
Mean HI antibody titers (Log 2) of pathogenicity test of the isolate 18B and 36 L with positive and negative control in white Hy-Line chicks (42-day-old) against antigens of H9, H5 avian influenza and Newcastle disease (ND).

Virus		HI titers pre-challenge (n = 5)						HI titers post-challenge (n = 5)					
		1	2	3	4	5	Mean	1	2	3	4	5	Mean
AIV-H9	**G1**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	G2*	0.0	0.0	0.0	0.0	0.0	0.0[b]	5.0	4.0	6.0	3.0	6.0	**4.8**[a]
	G3*	0.0	0.0	0.0	0.0	0.0	0.0[b]	7.0	6.0	8.0	6.0	7.0	**6.8**[a]
	G4*	0.0	0.0	0.0	0.0	0.0	0.0[b]	4.0	3.0	7.0	5.0	6.0	**5.0**[a]
AIV-H5**		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NDV**		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Means with different letters within the same row were significantly different at $P \leq 0.05$.
* Random blood samples were taken from different subgroups in each group.
** All groups (G1 to G4) showed negative results (0.0) for HI-anybody titers against AIV-H5 and NDV antigens pre- and post-H9-challenge.

compared to the results of Afifi et al. [8] on the commercial flocks which the seroprevalence ranged from 11.1% in broilers to 72.2% in layers and 58.3% in the breeder flocks. According to the results of seroprevalence in our study, the H9-AIV is also endemic in backyard chickens using the prepared antigen of the broiler isolate of high pathogenicity in HI test. This is the point of rationale between the seroprevalence and molecular analysis with pathogenicity in this study.

Seroprevalence of H9-AIV in backyard ducks in our study was 12.2% and HI titers ranged from 0.66 to 4.33 log2. The reason of the low seroprevalence in ducks is that the households slaughter the birds as soon as any disease condition starts to appear or they sell them in live bird markets (LBM) instead of losing them. Ducks have a binding preference for Sia (α2-3) Gal-terminated receptors that found in duck's intestinal tract where Sia (α2-6) Gal-terminated receptors is absent which explains this preference [25]. Matrosovich et al. [26] found that the H9 avian influenza virus preferentially binds to Sia (α2-6) Gal-receptors, for this reason; we can explain the low seroprevalence of ducks in our results. Moreover, Perkins and Swayne [27] compared the AIV titers in both chicken and duck tissue and reported that ducks had lower titers than chickens. It is worth to mention that some genotypes of H9-AIV, especially genotype B and F are effectively adapted for replication in ducks than others as genotype A which in turn has an effect on the virus transmission between birds [28].

In this study, the phylogenetic analysis showed the placement of both isolates within G1B, the same lineage circulating in the Middle East as previously shown [29]. HA has its role in the pathogenicity of influenza virus at the level of the cleavage site [30]. In the present study, partial HA sequencing was performed. Firstly, the cleavage site sequence was PARSSR/GLF for both isolates, the typical for LPAI which cleaved by trypsin-like protease localized in certain organs in the body of birds [31]. The amino acid sequence analysis of both isolates in this study revealed 10 amino acid substitutions suggesting some sort of antigenic drift, which helps the virus to escape from the immune system of the host [32]. The partial sequence analysis of HA revealed that HA1 genes share similarity 98% with each other and 98–99% with both first isolated strains, the first Egyptian quail isolate in 2011 [29] and the first Egyptian chicken isolate in 2012 [33]. No available previously reported literature to contribute to influenza virus H9N2 pathogenicity in these observed amino acids sequence alterations in the HA1. Meanwhile, influenza virus variation is a feature of influenza A virus [34].

In this field study during sampling, we found that with any other viral or bacterial pathogen exacerbates the disease condition with H9 infection, which increases the mortality rate up to 80% [35]. The co-infections of *Staphyloccocus aureus*, *Haemophilus paragallinarum* [2], flagellated bacteria [3], Infectious bronchitis virus (IBV) or live vaccine [2,35], Infectious laryngeotracheitis virus (ILTV) vaccine [36] and AIV H5N1 [37] exacerbate H9N2 infection that cause systemic infection, increased pathogenicity and virus shedding titers, poor weight gain, high rates of mortality, vaccination failure and severe economic losses in poultry flocks. The alteration in tissue tropism occurs due to the co-infection which the bacterial proteases could cleave the H9-AIV [1]. The stimulation of the host cell to secrete more proteases increases the pathogenicity of H9 [3]. Although the importance of HA cleavability for the spread of infection is recorded, the tissue tropism of one strain is not essentially applicable to the others [38].

In our study, interestingly, the pathogenicity index (PI) of H9N2 broiler strain in the inoculated experimental chicks ranged from 1.2 with 10^6 EID_{50} to 1.9 with $10^{7.5}$ EID_{50}. This PI indicated a highly pathogenic effect, with high mortality (up to 100%) in the inoculated chicks correlated with the high mortality (80%) in the flock where the virus was isolated. Meanwhile, H9N2 viruses do not commonly show such high pathogenicity indices. The high mortality rate can be explained by the virus itself or the stress factors, even during the inoculation and dealing with birds. The stress is one of the most important host factors which may increase the mortality up to 97% [1].

In the present study, the pathogenicity indices were differed by the inoculation of different doses, intravenous or oculonasal routes and H9N2 concentrations. It was assured that the signs of viral disease vary according to the dose and concentration of the virus; even with LPAI H9N2 virus [4]. In this study, the different inoculation doses of the broiler strain H9N2 virus of the same EID_{50} greatly affect the lesions and clinical signs. Similar results were previously obtained [39]. Moreover, the clinical signs of experimental infection with pneumotropic H9 avian influenza strains were more exacerbated through the intranasal route [5].

This pathogenicity study had drawn a special attention to the broiler isolate for 2 reasons. Firstly, at the field history, it found to cause severe respiratory manifestations and approximately 80% mortality in the broiler flock. Secondly, amino acid sequence revealed 10 amino acid substitutions in the HA gene. Surprisingly, the results of the pathogenicity test reinforced our doubts with a mortality rate up to 100% with inoculation of high doses and concentration of H9N2 infection. In our experiment, the pathogenicity index ranged from 1.9 in higher dose and concentrations of the virus to 0.8 in lower dose; similar to the isolate was used before [40].

Cyanosis in comb and wattle and also subcutaneous haemorrhage in the leg shank were observed (first record on AIV-H9N2 experimental infection) during the pathogenicity test in the broiler isolate, in this study, as a shocking observation, which could be explained by the virus replication in the endothelial lining of blood vessels as previously stated on H5-AIV [1]. In our study, the low pathogenicity AI-H9N2 of layer isolate was indicated from the low mortality and pathogenicity index (less than 1.2) as well as the clinical signs as sneezing, rales, nasal/ocular discharge, conjunctivitis and head swelling, while congested trachea and lung and swollen kidney were shown in lesions as previously described [36].

The tissue tropism of H9-AIV involved respiratory, urinary, lymphoid and digestive systems which were obviously detected in our

study post-challenge on post-mortem lesions of birds in the form of congested trachea and lung, haemorrhages in the small intestine and pancreas, swollen kidneys, enlarged spleen, petechial haemorrhage in bursa of fabricius, also cyanosis of combs and wattles of few birds. Moreover, these observations were similar to lesions reported in infected chickens with H9N2 [41]. In this study, the gross lesions, histopathological changes, and immunohistochemical staining also confirmed that the virus distributed systemically in the body organs, which agreed with the results of Mosleh et al. [41]. Our microscopical lesions of infection with H9N2 in the respiratory, urinary and lymphoid organs were previously confirmed [1,39,42]. Therefore, the results of the affected immune organs (spleen and bursa of Fabricius) confirmed the depressive effects of H9N2 on the humoral and cellular immunity.

Immunohistochemical findings revealed the presence of virus in trachea, lung, liver, spleen, kidney and intestine. A number of studies are available that shows the detection and lesions of various LP AIV isolates in trachea, lung, liver, kidney, and spleen of the commercial broiler and layer chicks by IHC staining which identified as dark brown deposits in the nuclei of pulmonary epithelial cells and within nuclei or cytoplasm of necrotic renal tubular epithelium in kidneys [43–48].

5. Conclusion

In conclusion, the H9N2 virus infection is currently circulating and endemic in both commercial and backyard poultry flocks in Egypt. Moreover, as a first report, the molecular analysis and pathogenicity of the tested strains confirmed a sort of high pathogenicity of H9N2 virus that requires continuous monitoring. To the best of our knowledge, this is one of a few published articles about the serosurvey of H9N2 in the backyards and the chickens in these backyards are higher in seroprevalence than ducks.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.02.011.

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