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RESEARCH ARTICLE

Studies on Some Respiratory Viruses Isolated from Chicken Flocks Suffering from Respiratory Troubles in Egypt

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Abstract

Increased respiratory infections in Egyptian chicken flocks in the last few years with variable mortality and increased economic losses urged us to study the viral causes of this problem. We examined 485 birds (360 broilers, 114 layers, and 11 breeders) from 100 chicken flocks suffered from respiratory troubles in Sharkia Governorate, Egypt during 2018-2019. The examined flocks were isolated in embryonated chicken eggs (ECGs) and examined by hemagglutination test (HAT). Fifteen flocks (12 out of 63 positive HA flocks and 3 out of 37 negative HA flocks) were identified using real time reverse transcriptase-polymerase chain reaction (rRT-PCR). The selected flocks were suffering from severe respiratory symptoms with high mortality and/or dropped egg production. The results revealed that 6 samples out of 12 positive HA were positive for highly pathogenic avian influenza virus (HPAIV-H5N8) and 6 samples were mixed infection by HPAIV-H5N8 and NDV. In contrast, one sample out of 3 negative HA was positive for infectious bronchitis virus (IBV). Conventional polymerase chain reaction (cPCR) was carried out for partial gene sequencing on five samples {2 AIV (HA gene), 2 NDV (F gene) and one IBV (SP1 gene)} to detect the genetic diversity of these viruses. The phylogenetic analysis revealed that H5N8, NDV and IBV isolates were related to other strains with nucleotide homology 91.8% -99.3%, 78.9%–98.9% and 71.6%–98.0% respectively. Experimental study was done on 40 one-day-old Ross chicks to evaluate vaccines against HPAI-H5N8 (A/Chicken/Sharkia/MS001/2018). The results revealed that the protection using vectored vaccine was 40% while inactivated vaccine was 20%. Also virus shedding decreased in inactivated vaccine than vectored vaccine. In conclusion, we recommend poultry keepers to use both of vectored and inactivated vaccines for perfect protection against HPAIV H5N8.

Keywords: real time reverse transcriptase-polymerase chain reaction (rRT-PCR) - highly pathogenic avian influenza virus (HPAIV-H5N8) - phylogenetic analysis - hemagglutination test (HAT) - virus shedding

Introduction

At the global level, chicken meat and eggs are considered one of the most consumed protein sources of animal origin. The poultry industry in Egypt has a significant influence on the Egyptian economy which reflects its importance especially after increasing of costs and high losses in the last few years. However, the risk of infectious diseases threatens the poultry industry constantly especially viral infections which are considered the most prevalent hazard in the poultry industry in Egypt. One of the most dominant problem in poultry farms are respiratory diseases which represent a large problem because of their multifactorial nature, as well as increasing respiratory disease outbreaks with various mortalities and different clinical signs have been reported in Egyptian commercial chicken flocks. [1]. Therefore, the poultry viruses have a great economic importance including the avian influenza virus (AIV), virulent Newcastle disease virus (vNDV) and infectious bronchitis virus (IBV) which lead to high mortalities in different chicken flocks [2, 3]**.** These three pathogens are able to induce

disease either independently or with mixed infections [1].

The highly pathogenic AIV (H5N8) was observed in Chinese flocks for the first time at 2010 [4], while the first appearance of HPAIV (H5N8) in Egypt during surveillance of AI at the end of 2016 revealed in wild birds belonged to clade 2.3.4.4b [5] and then the virus spread to the domestic birds, leading to many outbreaks in commercial poultry flocks causing high economic losses in the poultry industry [6]. In some vaccinated flocks, avian influenza (AI) was recorded in a respiratory disease complex syndrome causing high economic losses [2]. Avian Influenza virus (AIV) was the main cause in many respiratory disease troubles and decline in egg production which affecting both laying hens and broilers worldwide [7].

In many countries, although various strict vaccination programs, the poultry production has been threatened by NDV which is a very serious problem in spite of enormous efforts for controlling the disease have been made. The trading of poultry and poultry products with Middle Eastern countries and China was thought to be the cause of spreading of NDV genotype VII in Egypt [8, 9]. NDV and AIV are incriminated in various respiratory and egg production troubles which affect both broilers as well as laying hens worldwide [7]. In Egypt the outbreaks affected various poultry farms between 2011 and 2012 because of infection with NDV strains that confirmed to belong to class II, genotype VII [10].

Also infectious bronchitis virus (IBV) is an acute, highly contagious virus affecting upper respiratory system of chickens [11]. IBV has a great economic importance to the poultry industry because of the high morbidity and production losses which associated with the infection with IBV. The clinical signs including coughing, tracheal rales, sneezing, nasal exudate and also poor weight gain in broilers, while decline in egg quantity and poor egg-shell quality was recorded in layer and breeder flocks. In addition, the infected birds become more susceptible to secondary bacterial infections such as Escherichia coli [12]. In Egypt, IB was first described several reports [13, 14] emphasized the prevalence of the disease. In respiratory disease outbreaks, the clinical signs and postmortem lesions varied according to the strain of infecting virus, vaccination program, and whether single or multiple infections. Loss of productive performance of both egg and meat-type chicken flocks lead to percussive economic losses [15]. The main clinical manifestations were respiratory distress (tracheitis, with caseation at tracheal bifurcation) and nervous manifestations in the form of head tilting. The main predominant pathological picture was congested viscera [16, 17].

Therefore this study aimed to detect the viral causes of recurrent respiratory infections in chickens by using recent techniques as rRT-PCR and gene sequencing as well as evaluation of vectored and inactivated vaccines against isolated HPAIV- H5N8.

Materials and Methods

Ethical approval

The sample collection and experiment were conducted in line with rules set by the Animal Welfare and Research Ethics Committee, Faculty of Veterinary Medicine, Zagazig University, Egypt

Field samples collection

A total of 100 chicken flocks from different localities at Sharkia governorates in Egypt were examined during 2018-2019. These flocks were suffering from variable mortality rates with respiratory symptoms and/or severe drop in egg production. History of investigated flocks (locality, age, total numbers, mortality of last 3-5 days from onset of signs and vaccination program) were recorded as well as clinical signs as well as postmortem examination for diseased and dead birds (Table 1).

Virus isolation on Embryonated chicken eggs (ECEs)

Specimens were collected from suspected tissues (lung, trachea, spleen and proventriculus) from each flock. Homogenization and centrifugation of samples were done. Isolation was carried out by inoculation in ECEs at 10 days old through allantoic cavity with 0.1 ml/egg. The embryonic mortalities and gross lesions were recorded.

Hemagglutination test (HAT)

The harvested allantoic fluids for the examined flocks were tested by rapid slide HAT, according to [18].

Table (1): Descriptive data and lesion score for selected Chicken flocks

F. No	Locality	Age	Total number	Breed	Score of respiratory signs and lesions									MB%	MT%	Egg Dr.%
					Resp. signs	Nasal disch.	Sinus.	Trcaheitis	F.P.C	F.P.H	Air sacul.	Cas. plug	Pneumonia			
3	Abo Kabir	65 d	5000	Baladi	+++	+++	+++	+++	-	-	-	-	+++	65	18	
14	Abo Kabir	48 d	5000	Saso	+++	+++	+++	+++	-	-	-	-	+++	60	15	
19	Fakous	29 d	3500	Cobb	+++	+++	+++	+++	-	-	-	-	+++	35	9	
30	Abo Kabir	33 d	12000	Saso	+++	+++	+++	+++	++	++	++	-	+++	30	8	
39	Fakous	30 d	6000	Saso	+++	++	+++	+	+++	+++	+++	+++	+++	25	6	
44	Fakous	25 d	5000	Hubbard	+++	++	++	++	+++	+++	+++	++	+++	20	4	
48	Abo Kabir	45 d	6000	Saso	+++	+++	+++	+++	++	++	++	-	+++	50	10	
55	Abo Hammad	28 d	3000	Arbor Acres	+++	+++	+++	+++	++	++	++	-	+++	60	8	
63	Awlad Sakr	48 d	5000	Saso	+++	+++	+++	+++	++	++	++	-	+++	35	9	
64	Abo Kabir	29 d	12000	Ross	+++	+++	+++	+++	-	-	-	-	+++	50	15	
79	Fakous	65 d	5000	Bovans pullets	+++	+++	+++	+++	++	++	++	-	+++	35	9	
80	El Husseinia	8 m	5000	Lohman	+++	+++	+++	+++	-	-	++	-	+++	25	10	40
84	Kafr Sakr	65 d	7000	Lohman pullets	+++	+++	+++	+++	-	-	-	-	+++	50	20	
95	Fakous	10 m	5000	Isa Brown	+++	+++	+++	+++	-	-	-	-	+++	60	35	70
100	Awlad Sakr	15 m	6000	Baladi Breeders	+++	+++	+++	+++	++	++	++	-	+++	20	4	40

F. No=Flock Number Resp. signs= respiratory signs Nasal disch.= nasal discharges Sinus.= sinusitis F.P.C= fibrinous pericarditis F.P.H= fibrinous perihepatitis Air sacul.= air sacculitis Cas. Plug= caseated plug at tracheal bifurcation MB%= morbidity percent MT%= mortality percent Egg Dr. %= percent of drop in egg production - = no lesion or signs + = mild lesion or signs ++ = moderate lesion or signs +++ = severe lesion or signs d= days m= months

Real time –reverse transcriptase – polymerase chain reaction for AIV, NDV and IBV

Genomic RNA extraction

Viral RNA was extracted from 15 harvested allantoic fluid (12 positive and 3 negative HA test) using QIAamp Viral RNA Mini Kit (QIAGEN) catalogue No. 52904 (Qiagen, Valencia, Calif., USA) and according to manufacturer's instructions [19].

Primers and probes used for real time PCR (RT-PCR):

QuantiTect probe RT-PCR catalogue No.204443. RT-PCR was performed using following primers (obtained from Metabion, Germany). AIV-H5 primer for hemagglutinin (H gene) were "H5LH1: ACATATGACTAC CCACARTATTCAG" "H5RH1: AGACCAGCT AYC ATGATTGC" "H5PRO: [FAM] TCWACA GTGGCGAGT TCCCTAGCA [TAMRA]" [20] and primers used for AIV-H9 (H gene) were "H9F: GGAAGAATTAATTATTATTGGTCGGTA C" "H9R: GCCACCTTTTTCAGTCTGACATT" "H9 Probe [HEX] AACCAGGCCAGACATTGCGAGTAAGAT CC [TAMRA]" [21]. The cycling conditions of primers and probes for both AIV- H5 and H9 were as the following: reverse transcription at 50˚C for 30 minutes (min.), primary denaturation at 95˚C for 15 min., secondary denaturation at 94˚C for 30 seconds (sec.), annealing at 54˚C for 30 sec., extension at 72˚C for 10 sec. and the number of cycles was 40 cycles.

The primers used for NDV fusion protein (F gene) were as the following: "F+4839: TCCGGAGGATACAAGGGTCT" "F-4939: AGCTGTTGCAACCCCAAG" "F+4894: [FAM] AAGCGTTTCTGTCTCCTTCCTCA [TAMRA]" [22]. The cycling conditions of primers and probes for NDV (F gene) were as the following: reverse transcription at 50˚C for 30 min., primary denaturation at 95˚C for 15 min., secondary denaturation at 94˚C for 30 sec., annealing at 52˚C for 30 sec., extension at 72˚C for 10 sec. and the number of cycles was 40 cycles.

The primers used for IBV spike protein 1 (SP1 gene) were as the following: "AIBV-fr: ATGCTCAACCTTGTCCCTAGCA" "AIBV-as: TCAAACTGCGGATCATCACGT" "AIBV-TM: (FAM-TTGGAAGTAGAGTGACGCCCAAACTTC A (TAMRA)" [23]. The cycling conditions of primers and probes for IBV (SP1 gene) were as the following: reverse transcription at 50˚C for 30 min., primary denaturation at 95˚C for 15 min., secondary denaturation at 94˚C for 30 sec., annealing and extension at 60˚C for 45 sec. and the number of cycles was 40 cycles.

Primers and probes used for conventional real-time PCR (cPCR):

Primers used for cPCR were supplied from Metabion (Germany). cPCR was performed for gene sequencing using following primers: AIV-H5 primers (H gene) were "H5-kha-1: CCT CCA GAR TAT GCM TAY AAA ATT GTC" "H5-kha-3: TAC CAA CCG TCT ACC ATK CCY TG" [24]. The cycling conditions of primers and probes were as the following: reverse transcription at 50˚C for 30 min., primary denaturation at 95˚C for 15 min., secondary denaturation at 94˚C for 30 sec., annealing at 56˚C for 40 sec., extension at 72˚C for 30 sec. final extension at 72˚C for 10 min. and the number of cycles was 35 cycles.

NDV primers (M and F genes) were as the following: "M2: TGG-AGC-CAA-ACC-CGC-ACC-TGC-GG" "F2: GGA-GGA-TGT-TGG-CAG-CAT-T" [25]. The cycling conditions of primers and probes were as the following: reverse transcription at 50˚C for 30 min., primary denaturation at 95˚C for 15 min., secondary denaturation at 94˚C for 30 sec., annealing at 50˚C for 40 sec., extension at 72˚C for 45 sec. final extension at 72˚C for 10 min. and the number of cycles was 35 cycles.

The IBV primers (SP1 gene) were as the following: "IBV-HVR1-2-FW: GTK TAC TACTAC CAR AGT GC" "IBV-HVR1-2-RV: GAA GTG RAA ACR AGA TCA CCA TTT A" [26]. The cycling conditions of primers and probes were as the following: reverse transcription at 50˚C for 30 min., primary denaturation at 95˚C for 15 min., secondary denaturation at 94˚C for 30 sec., annealing at

52˚C for 40 sec., extension at 72˚C for 45 sec. final extension at 72˚C for 10 min. and the number of cycles was 35 cycles.

The sequencing and phylogenetic analysis

The purified cPCR product was sequenced in Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA using a Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster city, CA). A comparative phylogenetic analysis was performed for each virus of sequences using the CLUSTAL W multiple sequence alignment program, version 1.83 of Mega Align module of Laser gene DNA Star software [27]**.** The phylogenetic comparisons of the aligned sequences were performed with the MEGA 5 [28].

Experimental design

Forty one-day-old Ross chicks were classified into 4 groups. The vaccination (type, dose and route), challenge date, dose and route of inoculation were described in (Table 2), the challenged virus was HPAI-H5N8 named (A/Chicken/Sharkia/MS001/2018) isolated from Ross broiler chicken flocks 29 day old. HPAI-H5N8 was chosen because of the most isolates from examined flocks with respiratory signs and high mortality. The infective dose was $0.2x10^6$ ml by oculo-nasal route at 28 days old. Serum samples were collected at 21, 28 days before challenge and 12 days post challenge to detect antibody titers using reference antigens (MEVAC H5N8 clade 2.3.4.4).

Table (2): Experimental design

Group number	Vaccination	Inoculation date	Dose of inoculation	Route of inoculation
1	Hatchery vaccine VECTROMUNE AI 0.2 ml subcutaneous at day 1	28 days	$0.2x10^6$ ml	Occulonasal route
2	Inactivated vaccine FLU FEND H5N3 0.5 ml subcutaneous at day 7	28 days	$0.2x10^6$ ml	Occulonasal route
3	Non vaccinated	28 days	$0.2x10^6$ ml	Occulonasal route
4	Non vaccinated	Non infected	----------	----------

Fourteen oropharyngeal swabs were collected at 3rd, 5th, 7th and 10th days post challenge (dpc) from all groups (3 swabs from each group were pooled as one sample) were collected on viral transport media consisting of 10% Glycerol, 200 Ul of Penicillin per mL, 200 mg of Streptomycin per mL, 250 mg of Gentamicin per ml, and 50 U of Nystatin per ml and kept at -80oC till examined by qRT-PCR for detection of virus shedding.

Result and discussion

Clinical and post-mortem examination:

The examined flocks suffered from decreased feed and water consumption with general signs of illness in addition to variable mortality rates ranged from 2-25% and severe respiratory symptoms such as sneezing, gasping (Figure 1A) with swollen sinuses and conjunctivitis. Whereas some flocks showed cyanosis of comb and wattles (Figure 1B) with hemorrhage on the legs. In layer and breeder flocks the drop in egg production was up to 70% with soft shell egg (Figure 1C) with respiratory symptoms. In some examined flocks we noticed nervous signs such as muscular tremors, wing dropping, twisting of the head and neck, circling and complete paralysis, these results agreed with Ramzy [29], Shehata *et al* [30] who reported that NDV and AIV also invade other tissues such as the central nervous system (CNS) and the gastrointestinal tract (GIT) so different lesions had been reported on organs other than the respiratory system.

The postmortem investigation revealed that presence of tracheitis, fibrinous air sacculitis, perihepatitis and some flocks showed caseated plug at tracheal bifurcation (Figure 1D). Other flocks showed congested subcutaneous blood vessels, congested carcass with septicemic

picture and petechial hemorrhage on tips of proventricular glands (Figure 1E), also congested, enlarged pancreas with necrotic foci and enlarged mottled spleen (Figure 1F). NDV, AIV and IBV have a predilection for the respiratory tract of chickens. The present results agreed with those of Ramzy [29] who reported that the main clinical signs due to respiratory infection were observed as oculo-nasal discharges, gasping, wheezing, and rales with various mortalities. Postmortem lesions that were observed with different range among investigated flocks included general congestion of the abdominal viscera, edematous hemorrhagic tracheal mucosa with mucopurulent exudates. Congested edematous lungs were noticed, petechial hemorrhages were found on epicardium and coronary fat of the heart, adipose tissue of the abdomen, mucosa of duodenum and proventriculus.

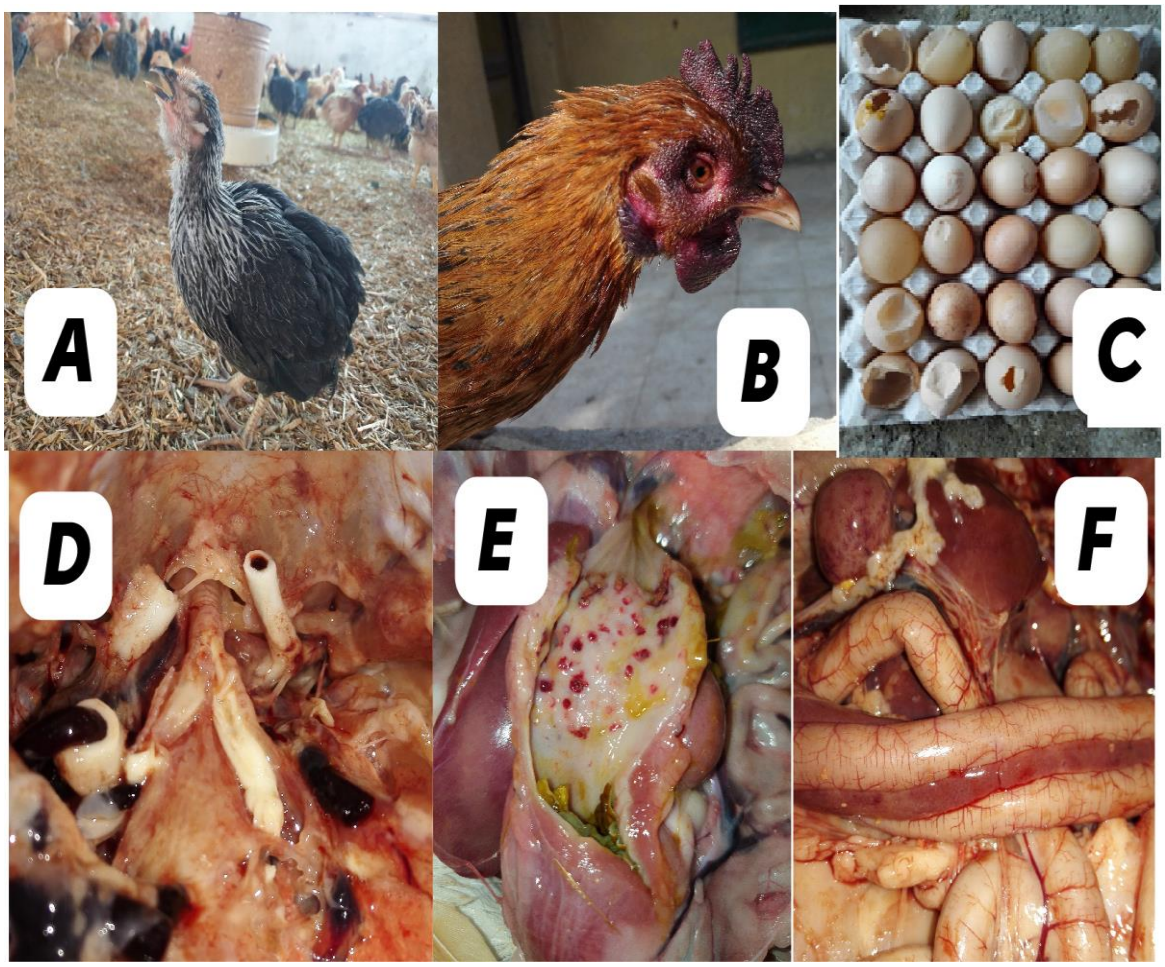

Figure (1): Different clinical signs and postmortem lesions on investigated naturally infected chicken flocks. (A): Sneezing, conjunctivitis and gasping of naturally infected flock that was positive by RRT-PCR for IBV. (B): Caseated plug at tracheal bifurcation of naturally infected flock that was positive by RRT-PCR for IBV. (C): Cyanosis of comb and wattles of naturally infected Baladi breeder flock that was positive by RRT-PCR for H5N8 and ND viruses' co-infection. (D): layer flock showing soft shell eggs and abnormalities in shape and color of eggs during natural co infection with H5N8 and NDV confirmed with RR-PCR. (E): Petechial hemorrhage on tips of proventriculus of naturally infected flock that was positive by RRT-PCR for H5N8 and NDV. (F): Congested, enlarged pancreas with necrotic foci and enlarged mottled spleen of naturally infected Arbor Acres broiler flock that was positive by RT-PCR for H5n8 and NDV co-infection.

Virus isolation and hemagglutination test

Tissue samples of examined flocks were isolated on ECEs for virus isolation, the embryos showed different degree of congestion and septicemia as well as curling and dwarfing were appeared on others.

The harvested allantoic fluids for all 100 flocks were subjected for HAT. The result of HAT revealed that 63 flocks (46 broilers, 15 layers and 2 breeders) were positive HA and 37 flocks were negative HA after first passage on ECEs. Fifteen flocks (12 out of 63 positive HA flocks and 3 out of 37 negative HA flocks) were selected for rRT-PCR. The selected flocks were suffered from severe respiratory symptoms with high mortality and/or dropped egg production. We suspected the cause of these troubles was one or more of respiratory viruses like as AIV, NDV and IBV in the most examined samples. These viruses were the main causes of respiratory infections and this result agreed with those of Awad *et* al. [10] who reported that AIV are common causes of respiratory diseases in chickens with economic importance worldwide. Also the spreading of H5N1 and the emergence of H5N8 viruses in Egypt caused in severe economic losses as reported by Selim *et al.* [5]. IBV was isolated from one broiler flock (30 days Saso flock) that suffered from severe respiratory signs with high mortality rate, similar result obtained by Ali *et al.* [31] who reported that many IBV outbreaks associated respiratory troubles with high mortality rate were attributed to the circulation of both classical and new nephro-pathogenic IBV variant strains.

Molecular identification by Real time RT-PCR test:

In the present study, as shown in (Tables 3, 4) the highest obtained results for respiratory viruses was AIV (12/15) of both single infection (6/15) 3 broilers and 3 layer flocks and mixed infection with NDV (6/15) 4 broilers, 1 layer and 1 breeder flocks, similar results were reported by Soliman *et al.* [32] who mentioned that the mixed infections of AIV and NDV was one of the most distressing problem of the poultry industry worldwide. The infection with some viruses is considered as a great stress to the birds and is considered as the main cause of co-infection and also it facilitated the infection with other viruses Arafat *et al.* [17], Samy and Naguib [33], Watanbe *et al.* [34].

Table (3): Results of virus detection by slide HA test and rRT-PCR on examined chicken flocks

	number of flocks examined by rRT-PCR	Positive HA result	Confirmed results with rRT-PCR		
			AIV only	AIV+NDV	IBV
Broilers	10	7/10	3	4	1
Layers	4	4/4	3	1	0
Breeders	1	1/1	0	1	0
Total	15	12/15	6/15	6/15	1/15

HA= hemagglutination test AIV only= single infection with AIV AIV+NDV= mixed infection with AIV and NDV

Table (4): Result of viruses' isolation in ECEs, HA activity, RT-PCR for selected chicken flocks

F. No	HA	Result of RT-PCR				Egg inoculation	
		H5	ND	IB	N8	Dead /total	Embryo lesions
3	+	CT 30	CT 30	-ve	CT 20	5/5	Congestion and PH*
14	+	CT 25	-ve	-ve	CT 21	4/5	Congestion and PH
19	+	CT 17	-ve	-ve	CT 14	5/5	Congestion and PH
30	+	CT 29	CT 26	-ve	CT 29	4/5	Congestion and PH
39	-	-ve	-ve	CT 29	nd	2/5	Curling and dwarfing
44	-	-ve	-ve	-ve	nd	3/5	Congestion
48	+	CT 31	CT 27	-ve	CT 30	4/5	Congestion and PH
55	-	-ve	-ve	-ve	nd	3/5	Congestion
63	+	CT 27	CT 27	-ve	CT 20	5/5	Congestion and PH
64	+	CT 17	-ve	-ve	CT 17	5/5	Congestion and PH
79	+	CT 31	-ve	-ve	CT 23	4/5	Congestion and PH
80	+	CT 26	-ve	-ve	CT 20	4/5	Congestion and PH

*PH: petechial hemorrhages -ve: No Ct after40 cycles

Sequencing and phylogenetic analysis

The result of phylogenetic analysis of partial sequences is shown in (Table 5) for the selected AIV isolates (for HA gene) showed that they belong to H5N8 highly pathogenic AIV clade 2.3.4.4.b (A/Broiler chicken/Egypt/MS001/2018) and (A/Layer chicken/Egypt/MS002/2019) with accession No. MW425876 and MW425877 respectively, our results agreed with Selim *et al,* [5] who mentioned that the first introduction of HPAI (H5N8) in wild birds in Egypt at the end of 2016 during surveillance of AI revealed that AI belongs to clade 2.3.4.4b (Figure 2). Our AIV isolates in this study were 100% identical to each other based on nucleotide and amino acid identities. Compared to other recently isolated Egyptian strains during 2016 to 2019, the identity ranged from 96.5% to 99.3%. While the identity percent was 91.8% to 98.2% when compared with other worldwide isolates.

The result of phylogenetic analysis of partial sequences for the selected NDV isolates (for F gene) showed that they belong to genotype VIIb (Figure 3) and they named (VIIb ch/Sharkia/MS006/2018) and (VIIb ch/Sharkia/MS007/2019) with accession No. MW429329 and MW429330 respectively. In this study, the two NDV isolates were 100% identical to each other based on amino acid and nucleotide identities. While the identity percent was 97.3% to 98.9% when compared to other recently isolated Egyptian strains in the last few years, while the identity percent was 78.9% to 79.4% when compared with vaccinal strains and was 82.1% to 97.6% when compared with other worldwide isolates. These results agreed with those of Awad *et al.* [10] Abdel-Monneim *et al.* [14].

Phylogenetic analysis of partial gene sequencing of one selected IBV isolate (Sp1 gene), it was related to classic strain (Figure 4) and named (IBV ch/Sharkia/MS009/2019) with accession No. MW429331**,** our results agreed with De Wit *et al.* [35] who reported that a number of local variants in addition to the widely known vaccine serotypes such as Mass and 4/91 strains circulate in Africa. The IBV isolate had close relationship to other Egyptian strains of 2001 to 2019 with nucleotide homology reached up to 76.5% to 98.0%, while the identity percent was 74.3% to 74.8% when compared with vaccinal strains and was 71.6% to 97.3% with other worldwide isolates.

Our results concluded that, the main respiratory diseases affecting broilers under Egyptian conditions were AIV, NDV and IBV, the environmental conditions and farm locality affected the incidence and spreading of avian viral respiratory diseases and continuous surveillance of these respiratory diseases that represent a large economic problem for poultry industry should be maintained to be on the center of the event to facilitate the possible control of these diseases and planning for prevention by selection of recent immunogenic vaccines.

Results of experimental infection

Challenge test was carried out with HPAIV-H5N8 for group 1, 2 and 3. The mortality percent during 12 days post challenge (dpc) was 60% in group 1 (vectored vaccine), 80% in group 2 (inactivated vaccine) and 100% mortality in group 3 (control non vaccinated group) within 6 dpc only, general signs of illness and typical signs of AI infection (cyanosis of comb and wattles, hemorrhage on shank, severe respiratory signs and greenish diarrhea) appeared in all infected groups but more severe in non-vaccinated group.

Antibody titers in group 1 were 4.1 log2, 3.75 log2 and 5.1 log2 at 21, 28 and 40 days old respectively, while in group 2 they were 4.6 log2, 3.5 log2 and 5.5 log2 at 21, 28 and 40 days old respectively. Whereas, in group 3 they were 2.3 log2, 1.5 log2 at 21 and 28 days old respectively. Finally, in the group 4 the antibody titers were 2 log2, 1.4 log2 and 0 at 21, 28 and 40 days old respectively.

Virus shedding carried out by using quantitative RT-PCR, the result revealed that virus shedding in group 1 was $1.701x10^3$, 4.632×10^3, $6.572x10^4$ and $4.332x10^5$ in 3^{rd}, 5^{th}, 7^{th} and 10^{th} dpc respectively, while in group 2 was 1.244×10^4, 1.389×10^4, $1.241x10^5$ and $1.737x10^3$ in 3^{rd}, 5^{th}, 7^{th} and 10^{th} dpc respectively and finally in group 3 was 4.043x105 and $5.030x10^6$ in 3^{rd} and 5^{th} dpc respectively. Virus shedding decreased in group 2 than group 1 and 3. Many HPAI viruses appear to be shed within a day or two in experimentally inoculated chickens Swayne and Beck [36] Van der Goat *et al.* [37] Swayne *et al.* [38] Bublot *et al.* [39]. Shedding of viruses was examined before the second day post challenge. However, some studies found that the chickens which inoculated intranasally could shed A/chicken/ Pennsylvania/1370/83 (H5N2), Swayne and Beck [36], Van der Goat *et al.* [40] and A/chicken/ Netherlands/ 621557/ 03 (H7N7), Van der Goat *et al.* [37] by day 1 pc in both respiratory secretions and feces.

Table (5): Accession numbers of the AIV, ND and IBV field isolates in this study on GenBank

Flock No.	Strain name on Gene Bank	Accession No.	Gene	Breed	Locality
64	A/Broiler chicken/Egypt /MS001/2018 (H5N8)	MW425876	HA	Ross	Abo kabir
84	A/Layer chicken/Egypt /MS002/2019 (H5N8)	MW425877	HA	Lohman pullets	Kafr Saqr
30	Avian orthoavulavirus 1 isolate MS006 fusion (F) gene	MW429329	F	Saso	Abo kabir
95	Avian orthoavulavirus 1 isolate MS007 fusion (F) gene	MW429330	F	Isa Brown	Fakous
39	Avian coronavirus isolate MS009 spike protein 1 (S1) gene	MW429331	S1	Saso	Fakous

Flock No: Flock number Accession No: Accession number

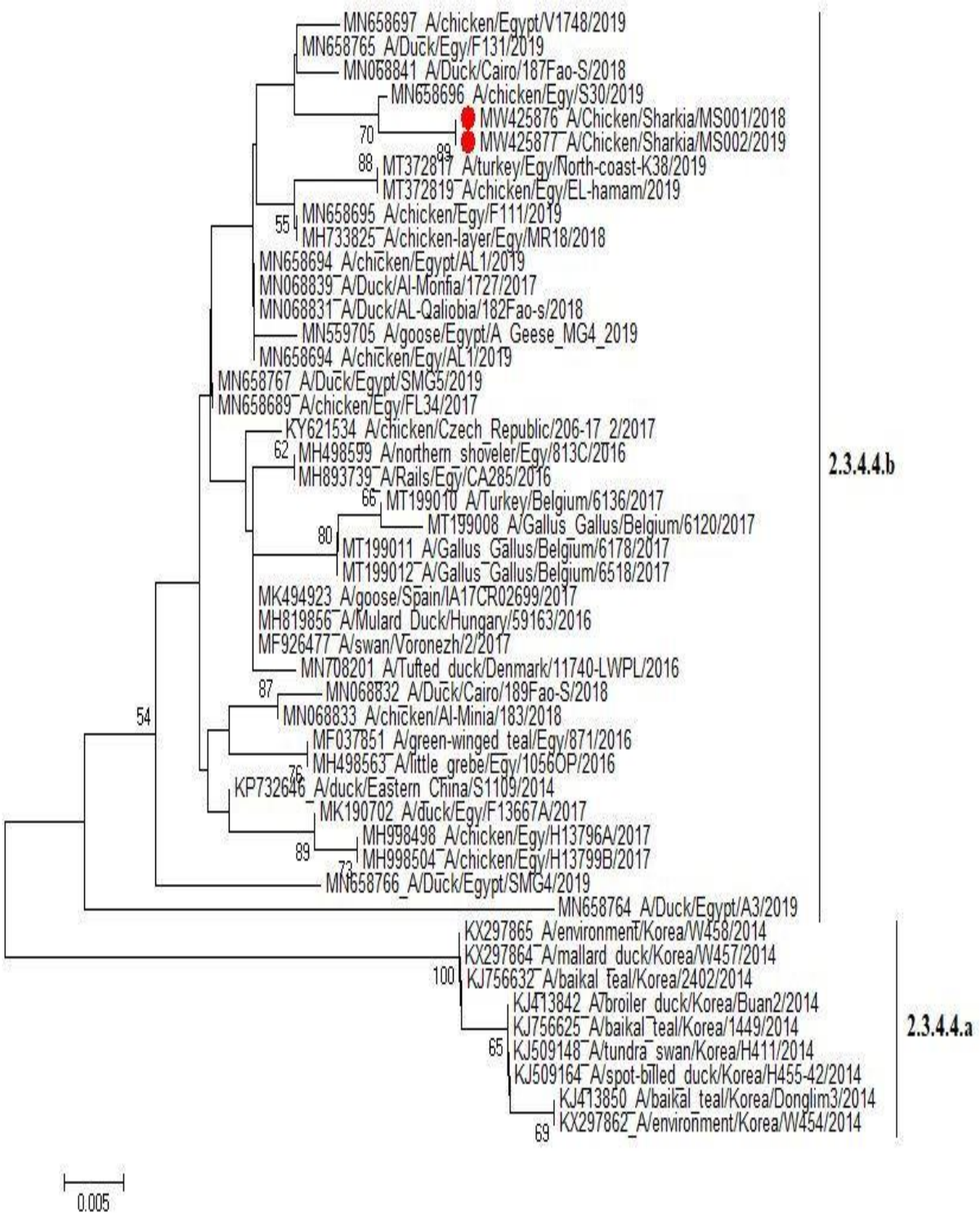

Figure (2): phylogenetic analysis of two isolated strains of H5N8 (red spots) with other published sequences.

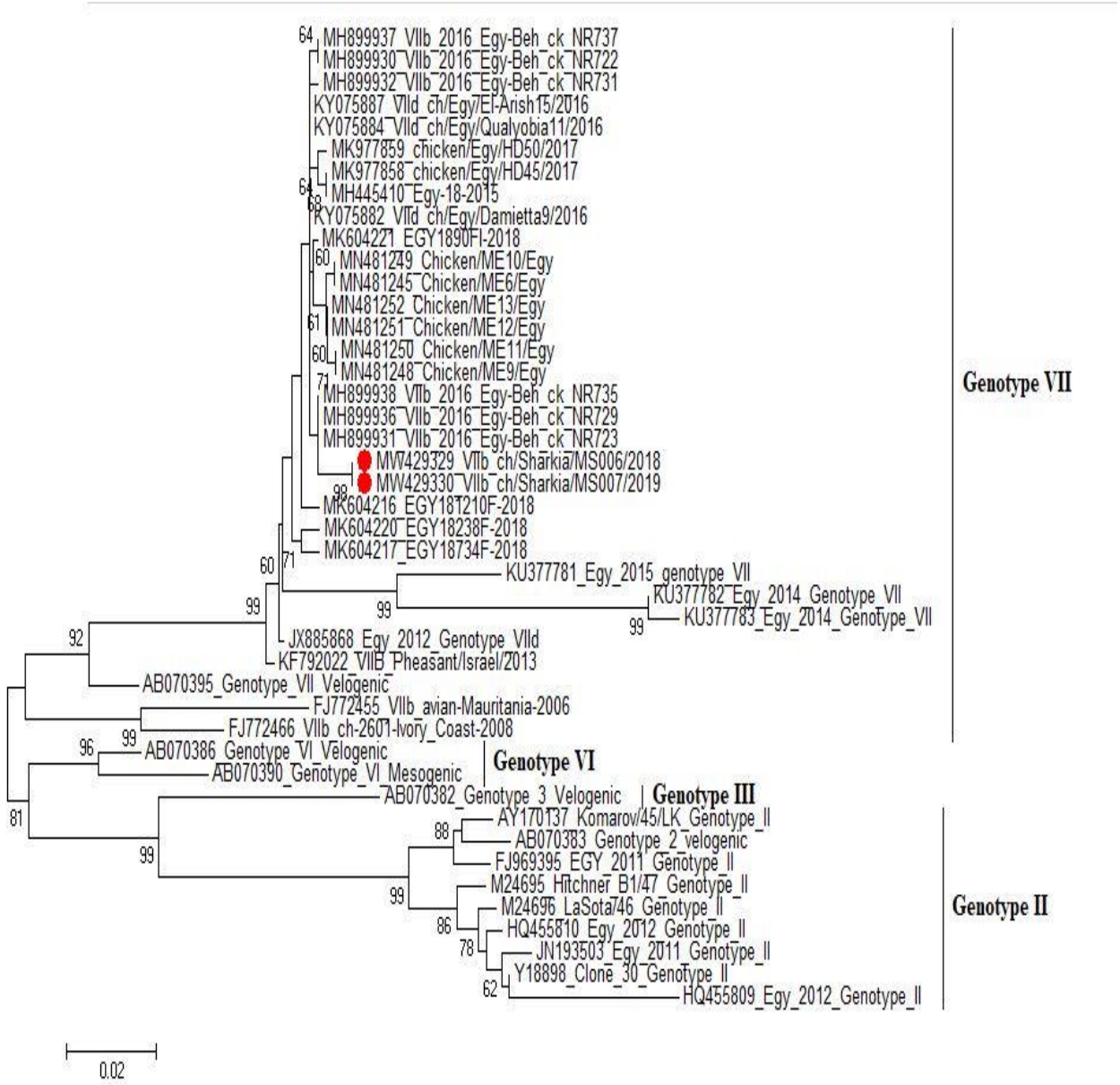

Figure (3): phylogenetic analysis of two isolated strains of NDv (red spots) with other published sequences.

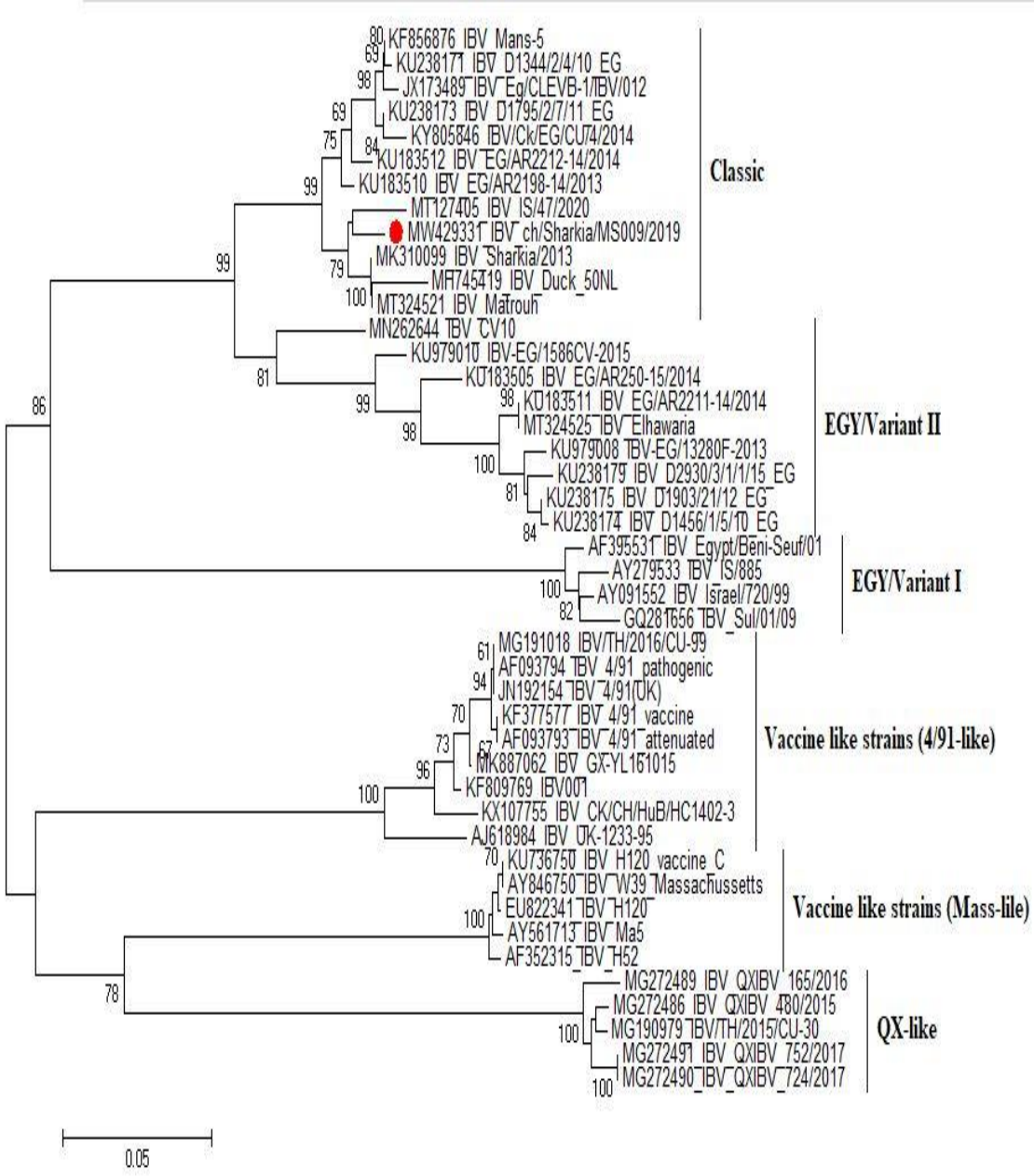

Figure (4): phylogenetic analysis of two isolated strains of IBv (red spot) with other published sequences.

Conclusion:

It is concluded that that AIV (H5N8), NDV (genotype VII) and IBV are highly infectious and considered the main causes of respiratory troubles in chickens leading to high mortalities and economic losses. From the obtained results, vaccine preparation should contain the new strains of AIV, NDV and IBV for complete protection of chicken flocks from recurrent respiratory infections.

Conflict of interest:

None of the authors have any conflict of interest.

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الملخص العربي

دراسات عن بعض الفيروسات التنفسية المعزولة من قطعان دجاج يعانى من مشاكل تنفسية فى مصر

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إن زيادة التهابات الجهاز التنفسي في قطعان الدجاج فى مصر في السنوات القليلة الماضية مع معدلات نفوق متغيرة وخسائر اقتصادية متزايدة دفعنا إلى دراسة الأسباب الفيروسية لهذه المشكلة. تم فحص 485 طائرا (360 طائر تسمين ، 114 طائر بياض ، و 11 طائر أمهات) من 100 قطيع دجاج كان يعانى من مشاكل في الجهاز التنفسي في محافظة الشرقية ، مصر خلال 2018-2019. تم عزل الفيروسات في بيض دجاج مخصب (ECGs) وفحصها بإختبار التلازن الدموي (HAT). تم إختيار 15 قطيع (12 من أصل 63 قطيع ايجابى لاختبار تلازن الدم HA و 3 من 37 قطيع سلبى لاختبار تلازن الدم) وتم تأكيد النتائج باستخدام تفاعل البلمرة المتسلسل (rRT-PCR). القطعان المختارة كانت تعانى من من أعراض تنفسية حادة مع ارتفاع معدل النفوق و / أو انخفاض شديد فى إنتاج البيض. أظهرت النتائج أن 6 قطعان كانت موجبة لفيروس أنفلونزا الطيور شديد العدوى (HPAIV-H5N8) وأن 6 قطعان كانت مصابة بعدوى مختلطة بفيروس أنفلونزا الطيور HPAIV-H5N8 و مرض نيوكاسيل NDV. في المقابل ، كانت عينة واحدة من عينات HA السلبية, كانت إيجابية لفيروس التهاب الشعب الهوائية المعدي (IBV). تم إجراء تفاعل البلمرة المتسلسل التقليدي (cPCR) لفحص التسلسل الجيني الجزئي على خمس عينات {2 AIV (جين HA) و 2 NDV (جين F) وجين IBV واحد (SP1)} لاكتشاف التنوع الجيني. أظهر التحليل الجيني أن معزولات H5N8 كانت متشابهة بسلالات أخرى ذات تماثل فى النيوكليوتيدات بنسبة 91.8٪ -99.3٪. بينما كانت نسبة تشابه معزولات النيوكاسيل 78.9%-98.9% ومعزولة IBV كانت 71.6٪ -98.0٪. أجريت العدوى التجريبية على 40 كتكوت روس عمر يوم واحد لتقييم اللقاحات ضد إنفلونزا الطيور عالية الخطورة / H5N8 (A / Chicken / Sharkia / MS001 (2018 ، وأظهرت النتائج أن الحماية باستخدام اللقاح المحمل كانت 40٪ بينما اللقاح الميت كان 20٪. كما انخفض إفراز الفيروس في اللقاح المعطل مقارنة باللقاح النواقل. في الختام ، نوصي مربي الدواجن باستخدام كل من اللقاحات المحملة واللقاحات الميتة للحماية الكاملة ضد فيروس HPAIV H5N8.