Research Article

Genetic Characterization and Pathological Evaluation of Clade 2.3.4.4b Avian Influenza Virus(H5N8) in Naturally Infected Domestic Ducks in Egyptian Farms

Sara Magdy Hashim[1], Elshaimaa Ismael[2], Mohamed Tarek[3], Faten Fathy Mohammed[1*], Fatma Amer Abdel Reheem[3], Rawhia Esawy Doghaim[1]

[1]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt; [2]Department of Veterinary Hygiene and Management, Egypt, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt; [3]Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Agriculture Research Center, Giza 12618, Egypt.

Abstract | The highly pathogenic avian influenza (HPAI) H5N8 virus causes severe economic deficit in the poultry production in Egyptian farms and has a potential intimidate for the public health, this subtype firstly was detected in Egypt in 2016 from wild birds. In the present study, samples were collected from 100 Egyptian domestic ducks of different ages from seven Egyptian governorates from period 2018 to 2020. Tissue specimens were allocated for HPAI H5N8 diagnosis using rRT-PCR and the results were 66 positive samples with the highest incidence in the Giza governorate. The winter season showed the highest incidence with 28 positive samples. For more characterization, the full HA gene was sequenced, followed by phylogenetic analysis according to the year examined, mortality rate, positive results of RT- qPCR with low Ct value (H5 and N8 were 15 and 20 respectively). The phylogenetic tree revealed that H5N8 field isolate clustering in clade 2.3.4.4b. The histopathological hallmarks, in H5N8 naturally infected ducks, included acute inflammatory, necrotic and vascular reactions that involve different tissues. The immunohistochemical characterization of viral antigen revealed direct relation between viral residence in tissue and developed pathology. The present study confirmed that H5N8 HPAI clade 2.3.4.4b, became a predominant strain during the period 2018-2020, causing severe outbreaks in duck farms in Egypt.

Keywords | Avian influenza, HPAI, H5N8, Ducks, Pathology, Egypt.

Received | August 30, 2022; **Accepted** | September 28, 2022; **Published** | November 20, 2022
***Correspondence** | Faten Fathy Mohammed, Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt; **Email:** fatenfathy21@yahoo.com
Citation | Hashim SM, Ismael E, Tarek M, Mohammed FF, Abdel Reheem FA, Doghaim RE (2022). Genetic characterization and pathological evaluation of clade 2.3.4.4B avian influenza virus(h5n8) in naturally infected domestic ducks in egyptian farms. Adv. Anim. Vet. Sci. 10(12): 2609-2621.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.12.2609.2621
ISSN (Online) | 2307-8316

INTRODUCTION

Avian influenza (AI) is an eminently contagious disease for poultry, that continue to disseminate in nature throughout the bird populations (Alexander et al., 2009), presenting a public health threat and economic loss associated with influenza pandemics (Van Wijhe et al., 2018). Avian influenza (AI) is caused by type A influenza virus, family Orthomyxoviridae, genus Influenza virus (Cox et al., 2000). The family Orthomyxoviridae comprises six general classified according to their serological cross-reaction to the nucleoprotein and matrix proteins (Bernard et al., 2013), Influenza virus A is known to affect birds naturally (Sonnberg et al., 2013). AIVs are classified into high and low pathogenic avian influenza virus (HPAIV and LPAIV) depending upon existing criteria agreed by the

World Organization for Animal Health (Capua and Alexander 2004; Mohammad & Mahmoud 2005). Recently, reassortment of avian influenza has been detected via acquiring neuraminidase (NA) genes from unrelated avian influenza viruses (Smith et al., 2015). China recorded an outbreak in a goose farm in 1996 at Qinghai Lake that caused by Asian H5 subtype, the virus has heam agglutination (HA) gene that originated from the H5N1 A/ goose/Guangdong/1/1996 (GS/ GD96) subtype (Xu et al., 1999).

The Asian H5 subtype shared the same HA gene, originated from the H5N1 (GS/ GD96) A/goose/Guangdong/1/1996 subtype, that caused an outbreak in Guangdong province at Qinghai Lake in 1996 in China (Xu et al., 1999).An emergence of a new clade of H5 viruses (2.3.4.4) was detected in 2014. This clades involving of viruses comprises the subtypes H5N1, H5N2, H5N3, H5N5, H5N6, and H5N8 (Adlhoch et al., 2014; Smith et al., 2015).

Avian influenza appeared to be the key pathogen of avian respiratory diseases in Egypt (Abdelwhab and Hafez 2011; Hassan et al., 2016), including highly pathogenic avian influenza virus (HPAIV) H5N1 of the Chinese origin (GS/GD96) goose/Guangdong lineage, that was first introduced into Egypt in 2006 and then the low pathogenic H9N2 (clade G1-B) that was introduced in 2011 (Aly et al., 2008), followed by HPAI H5N8 viruses of GS/ GD clade 2.3.4.4b introduction into Egypt in November 2016 via wild birds, causing further complications due to their spread into poultry industry (Hassan et al., 2020). The H5N8 AIV in Egypt was found to be closely related to the European H5N8 HPAI, clade 2.3.4.4b, based on the HA and NA sequences (Yehia et al., 2018). Egypt was the third country in the Middle East to report the H5N8 clade 2.3.4.4b (CIDRAP, 2016). AI viruses have been isolated from their natural reservoirs Charadriiformes and orders Anseriformes that include the domestic ducks (Alexander, 2007; Krauss et al., 2007). Ducks have played a role in novel influenza A viruses (IAVs) emergence events that threatened food security and public health, (McBride et al., 2021). Domestic ducks have been shown to be the reservoir of many avian influenza virus subtypes and allow their re-assortments, thus playing a role in virus ecology, propagation, and the emergence of new AIV genotypes (Barber et al., 2010; Parvin et al., 2020). Gross lesions in affected domestic ducks with HPAIVs exceedingly varied according to their geographical location and disease severity, depending on the host, virus pathogenicity and virulence, and the existence of the secondary micro-organisms. (Bröjer et al., 2009; Swayne et al., 2013).

A study that was applied in France to detect the pathogenicity of H5N8 in different birds, revealed that lesions were mild in ducks, in addition to inadequate detection of antigen in the respiratory and digestive linings. Also, it was found that leukocytes were lower in their viral antigenic load in ducks' tissue compared to that of chicken and guinea fowls (Gaide et al.,2022). Histopathology and immunohistochemical staining, in ducks affected by HPAI H5N8, revealed severe congestion and hemorrhages in whole organs, in addition to proliferation of glia cells and neuronal degeneration in brain, proliferative reaction with leucocytes infiltration in lungs and liver with degenerated and necrosed pancreatic acini (Abou-Rawash et al., 2012; Ruba et al., 2015; Mohammed et al., 2018). The present study aims to perform a survey on the incidence of AIVs subtype H5N8 infection in domestic ducks in Egyptian farms at different localities, phylogenetic analysis of the isolated strain, further characterization of pathological alterations developed in different organs of ducks infected by AIVs subtype H5N8 infection, and applying a correlation between histopathological lesions and residence of viral antigen in tissues using immunohistochemistry.

MATERIALS AND METHODS

Ethics statement

The present work procedures were approved by the Animal Care and Use Committee at Cairo University, Egypt (Approval number of ethics committee: Vet CU28/04/2021/285).

Sampling

In a survey conducted from May 2018 to January 2020, Samples were collected from 100 suspected domestic ducks of different ages (7 to 200 days old) and breeds (*Sudani, Pekin, Muscovy*), located in different Egyptian provinces (Giza, Fayoum, Sharqia, Behera, Dakahlia, Menia, , and Assiut*)* were examined against HPAI H5Nx virus. Postmortem examination for carcasses was obtained both from sick or moribund ducks that were suffering from mild to moderate respiratory and nervous signs as well as diarrhea. Sick ducks were selected and sent to the Reference Laboratory for Quality Control on Poultry Production (RLQP, Egypt), Animal Health Research Institute, Dokki, Giza, Egypt for real time Rt-PCR and sequencing. Data were recorded from all positive H5N8 naturally infected domestic ducks regarding their epidemiology, mortality rate and vaccination program as shown in Table 1 & 2.

Virus isolation and molecular characterization

Viral RNA was extracted from the collected tissue samples using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were tested against AIV H5N8 and H5N1 subtypes using RT-Qpcr specific for HA and NA segments (Hoffmann et al., 2016), using Stratagene MX3005P real

Table 1: Number and distribution of H5N8-positive rRT-PCR samples at different age among different duck breeds in different provinces between 2018-2020

Date	Governorate	Total no. of cases	Age (days)	Breed	Mortality Rate	Vaccination regime	
						Frequency	H5 Clade
2018-2020	Giza	43	60 - 200	Sudani Pekin Muscovy	21.2 %	2X - *8 samples are not vaccinated	2.2.1
2018-2020	**Sharqia**	21	7 - 200	Sudani Pekin	2.5 %	2X	2.2.1
2019	**Menia**	12	22 - 50	Sudani	11 %	1X	2.2.1 2.3.2
2018-2019	**Dakahlia**	9	150 - 200	Sudani Muscovy	15%	3X	2.2.1 2.3.2
2019	**Fayoum**	8	120 - 200	Sudani	3.6 %	3X	2.2.1 2.3.2
2019	**Assiut**	4	150 - 200	Sudani	3.5 %	3X	2.2.1
2019	**Behera**	3	160 - 200	Sudani	5 %	3X	2.3.2

*Samples were collected from clinically diseased or freshly dead birds.
* Reverse transcription quantitative Polymerase Chain Reaction were ranged from 15 – 30 (Considered positive less than 35 CT).

Table 2: Geographical distribution, incidence and seasonal incidence of H5N8 positive rRt-PCR in different breeds

Governorate	Total no. of cases	Incidence (%)	Seasonal incidence %				Breed		
			Winter	Spring	Summer	Autumn	Sudani	Pekin	Muscovy
Giza	43	33 **(76.7%)**	10/33 **(30.3%)**	18/33 **(54.5%)**	3/33 **(9.09%)**	2/33 **(6.06%)**	18/33 **(54.5%)**	11/33 **(33.3%)**	4/33 **(12.12%)**
Sharqia	21	16 **(76.1%)**	6/16 **(37.5%)**	_____	6/16 **(37.5%)**	4/16 **(25%)**	13/16 **(81.25)**	3/16 **(18.75%)**	_____
Menia	12	8 **(66.6%)**	8/8 **(100%)**	_____	_____	_____	8/8 **(100%)**	_____	_____
Dakahlia	9	3 **(33%)**	2/3 **(66.6%)**	1/3 **(33.3%)**	_____	_____	2/3 **(66.6%)**	_____	1/3 **(33.3%)**
Fayoum	8	3 **(37.5%)**	1/3 **(33.3%)**	1/3 **(33.3%)**	_____	_____	3/3 **(100%)**	_____	_____
Assiut	4	2 **(50%)**	1/2 **(50%)**	1/2 **(50%)**	_____	_____	2/2 **(100%)**	_____	_____
Behera	3	1 **(33.3%)**	_____	1/1 **(100%)**	_____	_____	1/1 **(100%)**	_____	_____
Total %	100	**66 (66 %)**	28 (42.4%)	22 (19.8%)	9 (13.6%)	6 (9 %)	47 (71.2%)	14 (21.2%)	**5 (7.57%)**

-time PCR. Allantoic fluid, 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECEs), inoculation was done for viral isolation according to the OIE manual (Naguib et al., 2017). The harvested allantoic fluids were tested against virus hemagglutination action by hemagglutinin assay and verified by using RT-qPCR.

Sequencing and Phylogenetic Analyses

The complete gene segment of the HA was amplified using primers previously described by Hoper et al. (2009). The gene-specific RT-PCR amplicons were further processed using the QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany) to obtain a purified PCR products . cycle sequencing using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). Purification of the products was performed using Centrisep spin column (Thermo Fisher, Waltham, MA, USA) and sequenced on an Applied Biosystems 3130 genetic analyzer, USA. Thereafter, the obtained sequence of the HA gene was assembled and edited using Seqscape® software for primary analysis of the raw data. (Tamura et al., 2013). Sequence established in the present study were submitted to GenBank. In addition, retrieval of genetic sequences from representative HPAI H5N8 viruses were performed from the NCBI and GISAID platforms. The nucleotide sequences were edited using Bio Edit (Hall 1999), and

alignment analyses were performed using MAFFT (Katoh & Standley, 2013). Phylogenetic analysis was performed by employing maximum likelihood methodology using MEGA X software (Tamura et al., 2013).

Pathological examination

Postmortem examination was done on different organs (Trachea, lungs, heart, liver, pancreas, intestine, spleen, kidneys and brain) that were collected from naturally infected domestic ducks. Tissue specimens were preserved in 10% neutral buffered formalin, and routinely processed, sectioned, and stained with hematoxylin and eosin (H&E) stain (Bancroft, J.D. and, Layton 2013).

Immunohistochemical studies

Hyperimmune serum was prepared against H5N8 AIV by series of injections in rabbits, as described by Horwitz and Scharff (1969). Three healthy rabbits of approximately 3 kg body weight were used, two rabbits were inoculated for hyperimmune serum preparation and one was used as control. The two rabbits were injected with two ml of avian influenza (AI) vaccine (*ME VAC H5N8*) subcutaneously (S/C) and this was repeated for 4 successful weeks, and on the fifth week, immunized rabbits were euthanized. Blood samples were collected for serum separation according to (Simpura, 1998). Titration of antibodies (Abs) were for haemagglutination inhibition (HI) activity using a 96-well microtiter system. A/Ck/Scot/59(H5N1) subtype of AIV was used as the test antigens. Preincubation of eight haemagglutinations (HA) units of viral antigen with two-fold serial dilutions of Abs for 30 min at room temperature, then adding 0.5% chicken red blood cells in phosphate-buffered saline. Calculation of antibody titer depending on the highest dilution that completely inhibited haemagglutination was done. Antibody purification was carried out using Magne_ Protein G Beads (Promega Corporation, Madison, Wisconsin, USA).

RESULTS

Clinical Findings

The prevalence of AI-H5Nx disease in domestic ducks was studied for a period of 3 years (May 2018 to January 2020) in seven governorates in Egypt (Giza, Fayoum, Beheria, Dakahlia, Sharqia, Menia, and Assiut), 100 suspected domestic ducks (Sudani, Pekin, Muscovy), of different ages, were examined for HPAI H5Nx virus. Clinical signs were variable. Most ducks died in good body conditions and some without showing any clinical signs. other ducks exhibited nervous signs including tremors of the neck, head pressing on the ground, associated with greenish-yellow diarrhea and respiratory distresses as shown in (Fig. 1). The information regarding age, vaccination, symptoms manifested, and mortality rate were summarized as shown in (Table 1).

Figure 1: Clinical signs of HPAIV H5N8 infected ducks, (a) Sudani duckling, 60 days old from Giza governorate, showing greenish diarrhea and respiratory distress. (b) Pekin ducks, 120 days old from Giza governorate showing nervous signs (torticollis) including tremor of neck.

Geographical distribution and seasonal incidence of HPAI H5N8

A total of 66 positive samples were detected in seven governorates in Egypt (Giza, Fayoum, Behera, Dakahlia, Sharqia, Menia, and Assiut), according to the geographical distribution, the highest incidence was detected in Giza with (33 samples), Sharqia with (16 samples) and Menia with (8 samples) respectively, while the lowest incidence was recorded in Fayoum with (three samples), Dakahlia with (two samples), Assiut with (two samples) and Behera with (1 sample). . Samples were collected in different seasons from seven Egyptian governorates. During winter, 28 samples with a ratio of 42.2% (28/66) were collected and shown to be H5N8 infected duck flocks. During spring 22 samples with a ratio of 19.8% (22/66), respectively. During summer nine samples with a ratio of 13.3% (9/58). During Autumn six samples with a ratio of 9% (9/6). The geographical distribution and seasonal incidence of H5N8 infection in duck farms were summarized in in Table 2 and shown in Fig. 2.

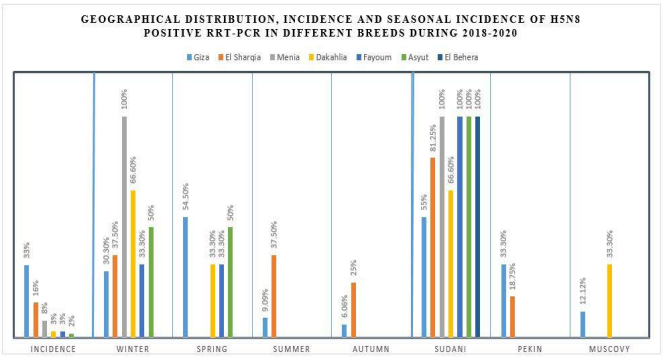

Figure 2: Geographical distribution, incidence and seasonal incidence of H5N8 positive rRt-PCR in different breeds during 2018-2020.

Table 3: Amino acid and Nucleotide sequences identity of HA gene of HPAI H5N8 isolates with HPAI H5N1 and HPAI H5N8 strains circulating in Egypt, other countries and avian influenza vaccines used in cross HI test during the study:

Nucleotide identity

Amino acid identity

	samples	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	31	32
1	A/duck/Egypt/F5/2006(H5N1)		90.3%	90.1%	90.9%	91.7%	90.3%	89.6%	90.7%	90.6%	90.5%	90.1%	90.1%	90.2%	90.3%	90.0%	90.1%	90.0%	91.3%	91.9%	90.5%	90.4%	90.3%	90.2%	90.1%	90.3%	90.1%	95.9%	89.3%	90.3%	96.2%	93.1%	91.1%
2	A/duck/Egypt/SS19/2017(H5N8)	90.3%		99.0%	98.6%	97.4%	99.7%	98.8%	98.8%	96.6%	99.0%	98.6%	98.8%	98.8%	98.7%	98.6%	98.7%	97.8%	96.0%	96.3%	98.9%	98.9%	98.8%	98.7%	98.3%	98.8%	98.2%	93.2%	97.4%	98.7%	88.2%	90.0%	96.1%
3	A/duck/Congo/17RS882-40/2017(H5N8)	90.1%	99.0%		98.8%	97.6%	99.2%	98.5%	99.1%	96.6%	99.2%	98.9%	99.0%	99.1%	99.0%	98.9%	99.0%	98.0%	96.0%	96.4%	99.2%	99.2%	99.0%	99.0%	98.6%	99.1%	98.5%	93.0%	97.7%	99.0%	88.1%	90.0%	96.2%
4	A/duck/Eastern China/S1109/2014(H5N8)	90.9%	98.6%	98.8%		98.5%	98.7%	98.0%	99.0%	96.4%	99.0%	98.7%	98.7%	98.9%	98.9%	98.6%	98.6%	97.7%	95.7%	96.3%	98.8%	99.0%	98.8%	98.6%	98.2%	98.8%	98.2%	93.7%	97.3%	98.9%	88.9%	90.2%	96.2%
5	A/breeder duck/Korea/Gochang1/2014(H5N8	91.7%	97.4%	97.6%	98.5%		97.5%	96.8%	98.0%	95.8%	97.8%	97.5%	97.6%	97.7%	97.6%	97.5%	97.4%	96.6%	94.9%	96.0%	97.7%	97.8%	97.6%	97.4%	97.0%	97.6%	97.0%	94.7%	96.2%	97.7%	89.7%	91.2%	95.3%
6	A/Duck/Ismailia/171Fao-SI/2017(H5N8)	90.3%	99.7%	99.2%	98.7%	97.5%		98.9%	99.0%	96.8%	99.1%	98.8%	98.9%	99.0%	98.8%	98.8%	98.8%	97.9%	96.0%	96.3%	99.0%	99.0%	98.9%	98.8%	98.4%	99.0%	98.4%	93.1%	97.5%	98.8%	88.2%	90.0%	96.2%
7	A/Duck/Giza/1814/2018(H5N8)	89.6%	98.8%	98.5%	98.0%	96.8%	98.9%		98.3%	96.0%	98.4%	98.1%	98.2%	98.3%	98.2%	98.1%	98.2%	97.2%	95.2%	95.6%	98.4%	98.4%	98.2%	98.2%	98.0%	98.3%	97.7%	92.6%	97.0%	98.2%	87.5%	89.2%	95.7%
8	A/Duck/Egypt/A3/2019(H5N8)	90.7%	98.8%	99.1%	99.0%	98.0%	99.0%	98.3%		96.8%	99.2%	99.0%	99.2%	99.2%	98.9%	99.2%	99.3%	98.5%	96.2%	96.8%	99.0%	99.1%	99.1%	99.3%	98.9%	99.4%	98.8%	93.4%	98.0%	99.0%	88.7%	90.3%	96.7%
9	A/Duck/Egypt/SA1/2020(H5N8)	90.6%	96.6%	96.6%	96.4%	95.8%	96.8%	96.0%	96.8%		96.6%	96.4%	96.4%	96.5%	96.5%	97.0%	97.0%	96.4%	94.2%	97.8%	96.4%	96.6%	96.4%	96.6%	96.1%	96.3%	96.6%	92.6%	95.8%	96.4%	88.9%	89.6%	94.4%
10	A/common teal/Korea/w/549/2016(H5N8)	90.5%	99.0%	99.2%	99.0%	97.8%	99.1%	98.4%	99.2%	96.6%		99.1%	99.1%	99.3%	99.4%	99.0%	99.0%	98.1%	96.2%	96.5%	99.2%	99.4%	99.2%	99.0%	98.6%	99.2%	98.6%	93.3%	97.7%	99.4%	88.4%	90.1%	96.3%
11	A/teal/Egypt /823C/2016(H5N8)	90.1%	98.6%	98.9%	98.7%	97.5%	98.8%	98.1%	99.0%	96.4%	99.1%		99.0%	99.2%	98.8%	98.9%	99.0%	98.0%	95.9%	96.4%	98.9%	99.0%	99.2%	99.0%	98.6%	99.1%	98.5%	92.8%	97.8%	99.0%	88.2%	89.6%	96.2%
12	A/tufted duck/Denmark/11740/2016(H5N8)	90.1%	98.8%	99.0%	98.7%	97.6%	98.9%	98.2%	99.2%	96.4%	99.1%	99.0%		99.2%	98.8%	99.0%	99.1%	98.2%	96.0%	96.4%	98.9%	99.0%	99.2%	99.4%	99.0%	99.2%	98.6%	92.9%	97.8%	98.8%	88.0%	89.9%	96.4%
13	A/Mulard Duck/Hungary/5916/2016(H5N8)	90.2%	98.8%	99.1%	98.9%	97.7%	99.0%	98.3%	99.2%	96.5%	99.3%	99.2%	99.2%		99.0%	99.0%	99.0%	98.1%	96.0%	96.6%	99.1%	99.2%	99.8%	99.2%	98.8%	99.2%	98.6%	93.0%	97.7%	99.0%	88.1%	89.8%	96.3%
14	A/Pekin duck/South Africa/17080481/2017(H5	90.3%	98.7%	99.0%	98.9%	97.6%	98.8%	98.2%	98.9%	96.5%	99.4%	98.8%	98.8%	99.0%		98.7%	98.8%	98.0%	95.9%	96.3%	99.1%	99.1%	99.0%	98.8%	98.4%	98.9%	98.3%	92.9%	97.6%	99.2%	88.2%	89.7%	96.3%
15	A/Duck/Egypt/FD189/2017(H5N8)	90.0%	98.6%	98.9%	98.6%	97.5%	98.8%	98.1%	99.2%	97.0%	99.0%	98.9%	99.0%	99.0%	98.7%		99.6%	98.8%	96.6%	97.0%	98.8%	98.9%	98.9%	99.1%	98.7%	99.5%	99.2%	92.7%	98.3%	98.7%	88.1%	89.6%	96.8%
16	A/Duck/Egypt/FAO-S78/2017(H5N8)	90.1%	98.7%	99.0%	98.6%	97.4%	98.8%	98.2%	99.3%	97.0%	99.0%	99.0%	99.1%	99.0%	98.8%	99.6%		98.8%	96.6%	97.0%	98.8%	99.0%	99.0%	99.2%	98.8%	99.6%	99.2%	92.8%	98.4%	98.8%	88.1%	89.7%	96.8%
17	A/Duck/Egypt/SMG6/2018(H5N8)	90.0%	97.8%	98.0%	97.7%	96.6%	97.9%	97.2%	98.5%	96.4%	98.1%	98.0%	98.2%	98.1%	98.0%	98.8%	98.8%		96.0%	96.3%	97.9%	98.0%	98.0%	98.2%	97.8%	98.6%	98.3%	92.1%	98.0%	97.8%	88.2%	89.7%	96.7%
18	A/Duck/Egypt/A3/2019(H5N8)	91.3%	96.0%	96.0%	95.7%	94.9%	96.0%	95.2%	96.2%	94.2%	96.2%	95.9%	96.0%	96.0%	95.9%	96.6%	96.6%	96.0%		94.8%	96.0%	96.2%	96.0%	96.2%	96.0%	96.6%	96.4%	92.4%	95.6%	96.0%	90.3%	89.6%	94.7%
19	A/Duck/Egypt/SMG5/2019(H5N8)	91.9%	96.3%	96.4%	96.3%	96.0%	96.3%	95.6%	96.8%	97.9%	96.5%	96.4%	96.4%	96.6%	96.3%	97.0%	97.0%	96.3%	94.8%		96.3%	96.4%	96.6%	96.5%	96.2%	96.9%	96.7%	93.5%	95.9%	96.2%	90.5%	90.1%	94.4%
20	A/chicken/Israel/1048/2016(H5N8)	90.5%	98.9%	99.2%	98.8%	97.7%	99.0%	98.4%	99.0%	96.4%	99.2%	98.9%	98.9%	99.1%	99.1%	98.8%	98.8%	97.9%	96.0%	96.3%		99.3%	99.2%	99.0%	98.6%	99.1%	98.5%	93.2%	97.6%	99.1%	88.4%	89.9%	96.2%
21	A/Cygnus olor/England/AS00918/2016(H5N8	90.4%	98.9%	99.2%	99.0%	97.8%	99.0%	98.4%	99.1%	96.6%	99.4%	99.0%	99.0%	99.2%	99.1%	98.9%	99.0%	98.0%	96.2%	96.4%	99.3%		99.3%	99.1%	98.7%	99.2%	98.6%	93.2%	97.8%	99.2%	88.5%	90.1%	96.4%
22	A/reverse genetic/France/RG1/2016(H5N8)	90.3%	98.8%	99.0%	98.8%	97.6%	98.9%	98.2%	99.1%	96.4%	99.2%	99.2%	99.2%	99.8%	99.0%	98.9%	99.0%	98.0%	96.0%	96.6%	99.2%	99.3%		99.2%	98.8%	99.2%	98.6%	93.1%	97.8%	99.1%	88.2%	89.9%	96.4%
23	A/chicken/Belgium/807/2017(H5N8)	90.2%	98.7%	99.0%	98.6%	97.4%	98.8%	98.2%	99.3%	96.5%	99.0%	99.0%	99.4%	99.2%	98.8%	99.1%	99.2%	98.2%	96.2%	96.5%	99.0%	99.1%	99.2%		99.2%	99.4%	98.8%	92.9%	98.0%	98.9%	88.1%	90.1%	96.6%
24	A/chicken/Moscow/94/2017(H5N8)	90.1%	98.3%	98.6%	98.2%	97.0%	98.4%	98.0%	98.9%	96.1%	98.6%	98.6%	99.0%	98.8%	98.4%	98.7%	98.8%	97.8%	96.0%	96.2%	98.6%	98.7%	98.8%	99.2%		99.0%	98.4%	92.7%	97.7%	98.5%	87.9%	89.7%	96.2%
25	A/turkey/Israel/184/2017(H5N8)	90.3%	98.8%	99.1%	98.8%	97.6%	99.0%	98.3%	99.4%	96.9%	99.2%	99.1%	99.2%	99.2%	98.9%	99.5%	99.6%	98.6%	96.6%	96.9%	99.1%	99.2%	99.2%	99.4%	99.0%		99.2%	93.0%	98.4%	99.0%	88.4%	89.9%	96.8%
26	A/chicken/Al-Minia/183/2018(H5N8)	90.1%	98.2%	98.5%	98.2%	97.0%	98.4%	97.7%	98.8%	96.6%	98.6%	98.5%	98.6%	98.6%	98.3%	99.2%	99.2%	98.3%	96.4%	96.7%	98.5%	98.6%	98.6%	98.8%	98.4%	99.2%		92.6%	98.0%	98.6%	88.1%	89.7%	96.7%
27	A/duck/Anhui/1/2006(H5N1)	95.9%	93.2%	93.0%	93.7%	94.7%	93.1%	92.6%	93.4%	92.6%	93.3%	92.8%	92.9%	93.0%	92.9%	92.7%	92.8%	92.1%	92.4%	93.5%	93.2%	93.2%	93.1%	92.9%	92.7%	93.0%	92.6%		91.8%	93.2%	93.0%	94.3%	93.1%
28	rg/A/chicken/ME-2018(H5N8)	89.3%	97.4%	97.7%	97.3%	96.2%	97.5%	97.0%	98.0%	95.8%	97.7%	97.8%	97.8%	97.7%	97.6%	98.3%	98.4%	98.0%	95.6%	95.9%	97.6%	97.8%	97.8%	98.0%	97.7%	98.4%	98.0%	91.8%		97.6%	87.5%	88.8%	96.3%
29	A/green-winged teal/Egypt/877/2016(H5N8)	90.3%	98.7%	99.0%	98.9%	97.7%	98.8%	98.2%	99.0%	96.4%	99.4%	99.0%	98.8%	99.0%	99.2%	98.7%	98.8%	97.8%	96.0%	96.2%	99.1%	99.2%	99.1%	98.9%	98.5%	99.0%	98.6%	93.2%	97.6%		88.3%	89.9%	96.2%
30	A/chicken/Egypt/D10552B/2015(H5N1)	96.2%	88.2%	88.1%	88.9%	89.7%	88.2%	87.5%	88.7%	88.9%	88.4%	88.2%	88.0%	88.1%	88.2%	88.1%	88.1%	88.2%	90.3%	90.5%	88.4%	88.5%	88.2%	88.1%	87.9%	88.4%	88.1%	93.0%	87.5%	88.3%		91.1%	89.9%
31	A/duck/Guangdong/S1322/2010(H5N1)	93.1%	90.0%	90.0%	90.2%	91.2%	90.0%	89.2%	90.3%	89.6%	90.1%	89.6%	89.9%	89.8%	89.7%	89.6%	89.7%	89.7%	89.6%	90.1%	89.9%	90.1%	89.9%	90.1%	89.7%	89.9%	89.7%	94.3%	88.8%	89.9%	91.1%		90.3%
32	A/chicken/Egypt/AL1/2019(H5N8)	91.1%	96.1%	96.2%	96.2%	95.3%	96.2%	95.7%	96.7%	94.4%	96.3%	96.2%	96.4%	96.3%	96.3%	96.8%	96.8%	96.7%	94.7%	94.4%	96.2%	96.4%	96.4%	96.6%	96.2%	96.8%	96.7%	93.1%	96.3%	96.2%	89.9%	90.3%	

Viruses isolation, identification and Genetic characterization.

According to the results of RT- qPCR, detection of HPAI H5N8 possessed only 66 positive samples out of 100. Virus isolation and propagation were done by intra-allantoic inoculation of 9-11 day old SPF ECE, then incubated at 37 °C for 3-5 days. The harvested allantoic fluids were tested for virus hemagglutination action by HA and HI with specific antisera against AI-(H5N8 and H5N1), NDV, and EDS virus, which gave positive results with H5N8 only, while gave negative results with NDV and EDS. One sample was selected for isolation from Giza governorate, A/Duck/Egypt/SA1/2020 (H5N8), according to the year examined, mortality rate, positive results of RT- qPCR with low Ct value (H5 and N8 were 15 and 20 respectively), and seasonal incidence. HA and HI were applied on the selected sample against H5N1, NDV and EDS, in addition to RT-PCR for H5N8, H5N1 and NDV, for more confirmation of the purity of the sample. The sequence was generated for HA gene segments for an Egyptian H5N8 isolate. The obtained sequence was submitted to the GenBank under accession number OM049467. The Molecular characterization revealed that the HA cleavage site of the isolate A/Duck/Egypt/SA1/2020(H5N8) had a multiple basic amino acid motifs "PLREKRRKR/GLF". which referred to HPAIV H5N8 clade 2.3.4.4b by phylogenic analysis (Fig.3), resembling the Egyptian isolates from 2016 – 2020 with no major genetic differences. The similarity between the isolates in this study was shown in (Table 3), H5N8 isolate showed a similarity between the other Egyptian isolates (2016 - 2019) ranging from (90.6% - to 97.8%) and this cluster includes other H5N8 strains recently isolated from Asia (Eastern China, Korea) and from Europe (Denmark, England and France) with amino acid identity percent with each other ranging from (95.5% - to 96.6%), and Russia (Moscow) with amino acid identity percent with each other (96.1%). The amino acid identity percent between the local vaccinal strains and the field isolates was ranging from (95.8% - to 96.4%). While H5N1 vaccinal strain showed relativity low distinct range ranging from (88.6% - to 92.6%).

Gross lesions

Gross lesions in affected ducks included generalized congestion involving trachea, lungs, heart, liver, pancreas, intestine, spleen, kidneys and brain). Necrotic splenitis and pancreatitis were evident. Individual cases showed air sacculitis and fibrinous pericarditis indicative for secondary bacterial infection. Hepatic congestion with distended gall bladder were observed (Fig.4).

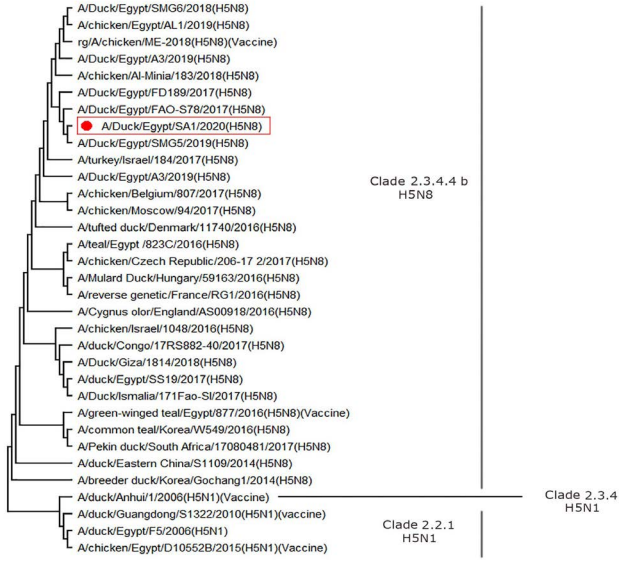

Figure 3: Phylogenetic relationship of HPAI H5N8 local field isolates to other selected AIV isolates based on nucleotide sequence of HA gene. Phylogenetic tree of the HA gene segment of HPAI H5N8 viruses. A phylogenetic tree including a total of 32 HA segments from different H5N8 viruses was obtained using MEGA X software.

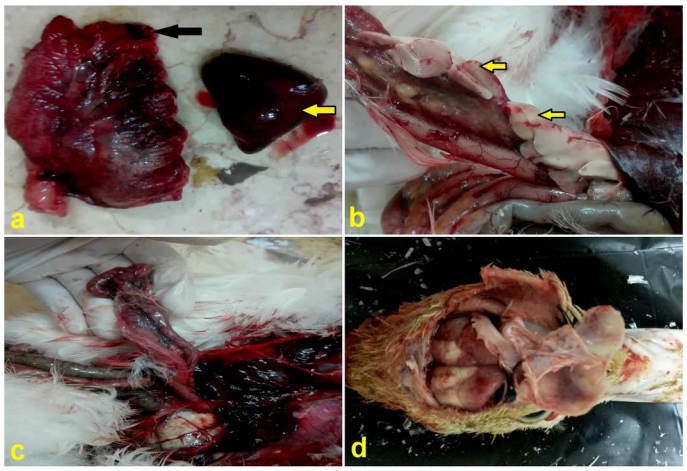

Figure 4: (a) Lung, duck showing congestion and edema (black arrow), spleen showing splenomegaly and congestion (yellow arrow). (b) Pancreas, duck showing necrotic areas (yellow arrows).) with hemorrhage (c) Duodenum, duck showing severe congestion of serosal vasculatures. (d) Brain, duck showing congestion.

Histopathological findings

The microscopic examination of tissues from AIV H5N8 infected ducks revealed severe histopathological alterations involving different organs. The histopathological alterations in the trachea were characterized by thickening of tracheal mucosa with deciliation, there was congestion blood vessels and interstitial edema. Generalized congestion of pulmonary vasculatures with perivascular edema, resulting in distension of interlobular spaces. The primary bronchi and parabronchus showed severe congestion of capillaries in parabronchi associated with focal mononuclear cell infiltration and hemorrhages (Figs. 5a &b). The heart showed remarkable histopathological changes, the sub-epicardial tissue showed marked thickening, edema and hemorrhage admixed with few mononuclear cells (Fig.5c). The cardiac myofibers in epicardial area showed intermuscular hemorrhage, edema and congested blood vessels (Fig.5d). Interstitial mononuclear cells infiltration was seen. Fibrinous pericarditis was detected in individual cases characterized by great thickening of the pericardium with eosinophilic fibrin and necrotic debris infiltrated by heterophils and macrophages. Regarding liver, the microscopic examination of liver from the affected cases revealed portal and sinusoidal congestion with vacuolization of hepatocellular cytoplasm (Fig.6a). Sinusoidal lymphocytosis was detected (Fig.6b). Necroapoptotic reaction of hepatocytes was evident (Fig.6c). Microscopic examination of pancreas revealed multifocal necrosis of its acini, dense eosinophilic cytoplasm with cell debris (Fig.6d), and lymphocytic cell infiltration in pancreatic acini. There was vacuolization and necroapotosis of exocrine acini were observed (Fig.6e). Congestion of the pancreatic blood vessels was detected. Histopathological examination of the duodenum showed severe ulcerative and hemorrhagic enteritis, characterized by hemorrhage at mucosal luminal surface, desquamation and necrosis on enterocytes, edema of lamina propria infiltrated by mononuclear cells. Severe congestion of blood capillaries in lamina propria extending into submucosa and serosa (Fig.6f). Necrotic splenitis was the main histopathological alteration in spleen of affected ducks. The spleen showed severe congestion of splenic vessels and sinusoids (Fig.7a). Diffuse lymphoid depletion and necrosis (Fig.7b) with fibrinoid necrosis and hyalinization of splenic ellipsoids were evident (Fig.7c). Reticuloendothelial hyperplasia, lymphocytolysis comprising the lymphoid follicles and bursal dependent lymphoid follicle were detected (Fig.7d). The kidney showed congestion of large vessels and peritubular capillaries associated with vacuolization and necrosis of renal tubular epithelium, accumulation of cellular and proteineous cast in tubular lumina were detected.

Concerning brain, the histopathological hallmarks are evident in cerebral vasculatures. Severe congestion and microthrombosis of blood vessels with endothelial capillary proliferation in the brain tissue were detected (Fig.8a), the lesion associated with spongiosis, encephalomalacia and gliosis of cerebral neuropil (Fig.8b). There was neuronal degeneration of large pyramidal neurons in cerebellar nucleus (Fig.8c). Picture of non-suppurative encephalitis was observed mainly in cerebral cortex , the lesion was characterized by vasculitis, lymphocytic infiltration in vascular wall, perivascular lymphocytic cuffing, neuronal degenera-

tion and microthrombosis (Figs. 8d - f).

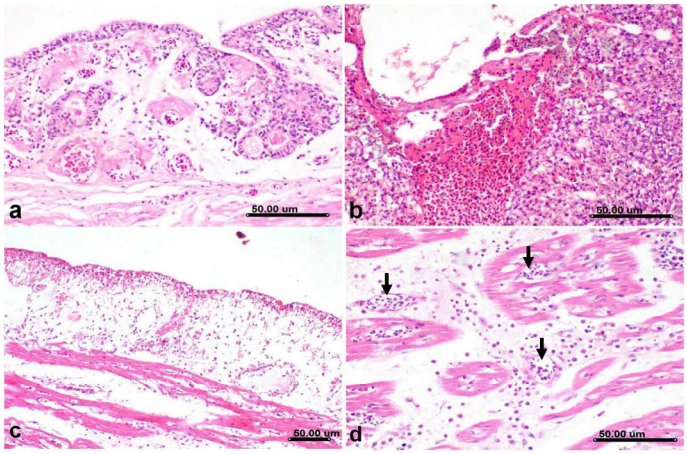

Figure 5: Histological sections of lung and heart from H5N8 infected duck, a) Primary bronchus showing deciliation of lining epithelium with congestion associated with interstitial edema and mononuclear cells infiltration. b) Parabronchus showing hemorrhage with proliferative reaction involving the air capillaries with mononuclear cells infiltration. c) Heart, epicardium showing desquamation of lining mesothelium associated with extensive subepicardial edema, congestion and mononuclear cells infiltration, note the extensive intermuscular edema of cardiac muscle fibers in the vicinity of subepicardial area. d) Myocardium showing necrosis of cardiac myocytes with mononuclear cells infiltration, congestion (arrow) and intermuscular edema. (Stain, H&E).

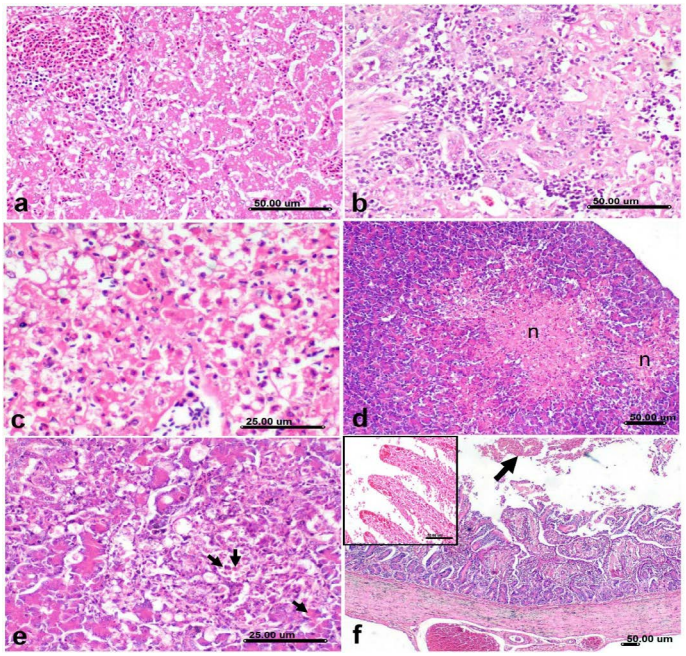

Figure 6: Histological sections of Liver, pancreas and duodenum from H5N8 infected duck, a) Liver showing severe congestion of portal vessels and hepatic sinusoids associated with vacuolization of hepatocellular cytoplasm. b) Liver showing lymphocytic infiltration of hepatic sinusoids. c) Liver showing severe necrotic reaction of hepatocytes with karyorrhexis and pyknosis of their nuclei d) Pancreas showing multifocal necrosis of exocrine pancreatic acini that replaced with eosinophilic necrotic tissue debris (n). e) Pancreas showing vacuolization of pancreatic acinar epithelium associated with apoptosis(arrow). f) Duodenum showing shortening, atrophy and blunting of duodenal villi, severe congestion of submucosal and serosal vessels and mucosal luminal hemorrhage (arrow), the inserted box in upper left corner showing severe ulceration of mucosa with congestion of blood capillaries in lamina propria that was infiltrated by mononuclear cells. (Stain, H&E).

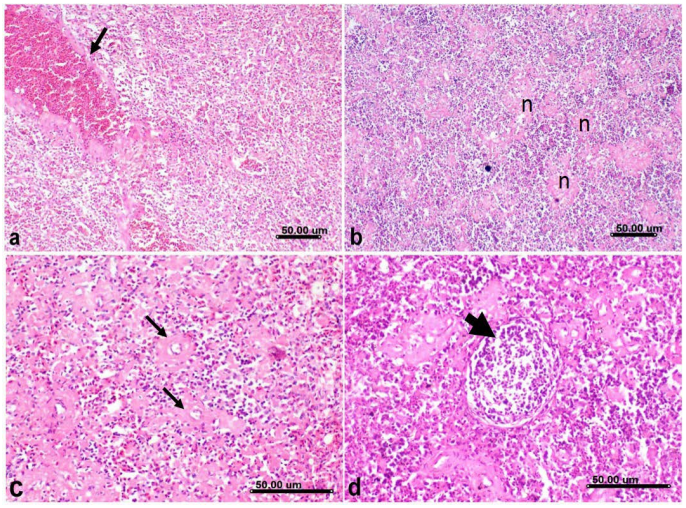

Figure 7: Histological sections of spleen from H5N8 infected duck, showing a) Severe congestion of splenic vessels (arrow) and sinusoids b) Spleen showing severe lymphocytic depletion with marked necrosis of lymphoid follicles (n). c) Fibrinoid necrosis of splenic arterioles (arrow). d) lymphocytic depletion and lymphocytolysis of lymphoid elements comprising the bursal associated follicles (arrow). (Stain, H&E).

Immunohistochemical Findings

AI antigen was detected in the different tissues. The expression of viral antigen was detected in tracheal mucosal epithelium and in capillary endothelium of parabronchial capillaries and pulmonary macrophages (Fig.9a). The cardiac myofibers showed strong expression of viral antigen (Fig.9b) in addition to antigen expression in vascular endothelium of large b.v (Fig.9c). Strong positive immune staining of viral antigen was detected in exocrine pancreatic acini and intestinal crypt epithelium of duodenum (Fig.9d). Positive staining of the scattered lymphocytes was detected in the spleen (Fig.9e), Further, expression of viral antigen was detected in cerebral vascular endothelium, neurons and glia cells (Fig.9f).

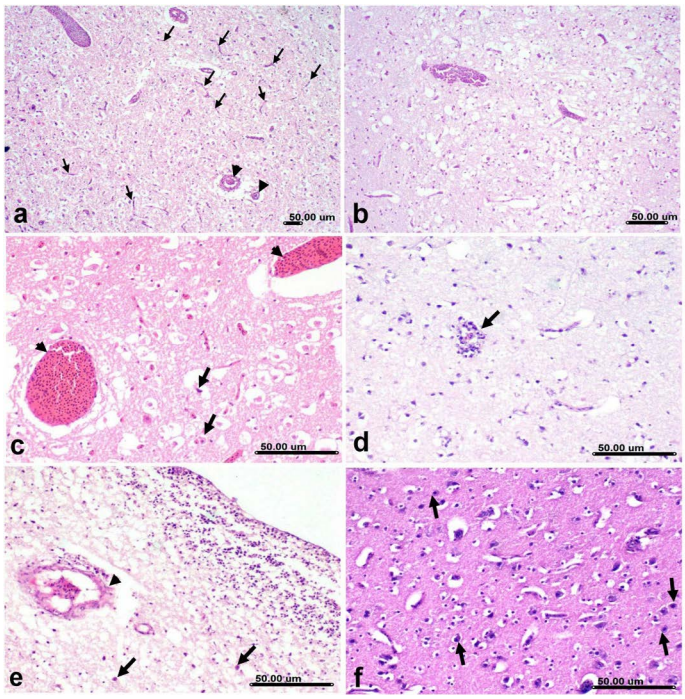

Figure 8: Histological brain sections from H5N8 infected duck showing; a) Congestion and micro- thrombosis of cerebral blood vessels (arrowhead) associated with capillary endothelial proliferation (arrow). b) Spongiosis and vacuolization of cerebral neuropil associate with diffuse gliosis. c) Marked congestion (arrowhead) associated with necrosis of pyramidal neurons involving the cerebellar nucleus(arrow). d) Vasculitis and perivascular lymphocytic cuffing (arrow) associated with gliosis. e) Microthrombosis (arrowhead) and hemorrhage in hippocampus associated with neuronal necrosis (arrow). f) Neuronal degeneration with neuronophagia associated with glisosis of cerebral grey matter. (Stain, H&E)

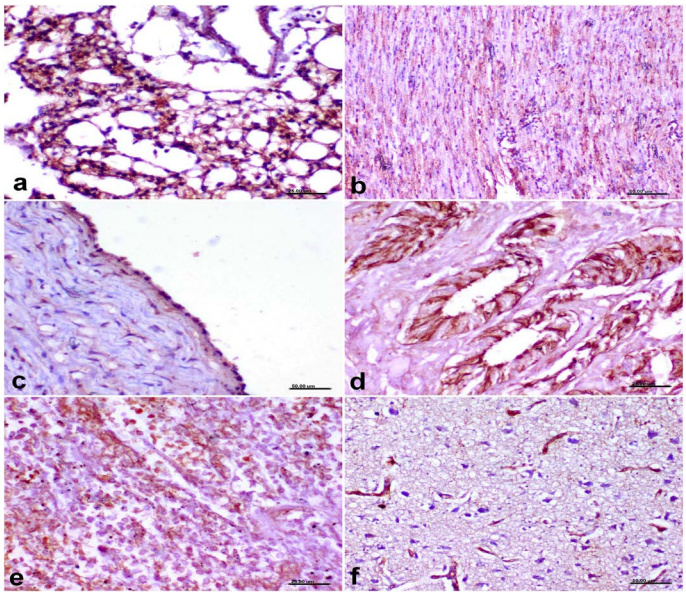

Figure 9: Histological immunohistochemical sections from H5N8 infected duck. a) Lung showing residence of viral expression in lining epithelium of air capillaries and in infiltrating inflammatory cells. b) Heart showing viral expression in cardiac myocytes. c) Large vessel showing expression of viral antigen in lining endothelium. d) Duodenum showing expression of viral antigen in epithelial lining crypts. e) Spleen showing residence of viral antigen in lymphocytes. f) Brain showing expression of viral antigen in neurons, Capillary endothelium and glia cells.

DISCUSSION

The present work aimed to study the incidence, genetic and pathological characterization of HPAI subtype H5N8 in naturally infected ducks in Egyptian farms between 2020-2021. Various surveys were conducted to estimate the prevalence of the disease in different governorates in Egypt. The High Pathogenic Avian Influenza (HPAI) is a contagious avian disease, causing significant economic deficit in the poultry production and a potential intimidate for the public health (Neumann, 2015). Domestic waterfowl can act as an intermediate key in the transmission of avian influenza among birds (Li et al., 2004), have been implicated in the dissemination and evolution of H5N8 HPAIV (Hill et al., 2015).

In the present study, most of the examined samples were collected from domestic ducks suffering from nervous symptoms with mild to moderate respiratory symptoms. Some die with high mortalities without producing any signs of clinical disease and these results agree with Kandeil et al. (2017), Anis et al. (2018) and Punnoose et al. (2021). Such observation was explained by Evseev & Magor 2019 who stated that ducks can limit the duration of the response against H5 HPAI infection, particularly of pro-inflammatory cytokine expression via induction of interferon type I and antiviral interferon-stimulated genes, including RIG-I pathway. Meanwhile Pohlmann et al. (2017) reported that most affected birds were found dead or exhibited severe clinical signs or sudden deaths. Punnoose et al. (2021) stated that H5 HPAIVs of Asian origin have evolved from insignificant or mild respiratory infections to some strains, including H5N8 clade 2.3.4.4b, causing severe clinical signs and lesions with high mortalities in ducks.

The present study revealed that Giza governorate had a higher infection rate than other governorates with a ratio of 33 %, while other governorates, Sharqia, Menia, Dakahlia, Fayoum, Assiut, and Behera recorded an infection ratio of 16%, 8%,3 %, 3 %, 2%, and 1%, respectively. Samples in our study were collected in different seasons. Based on that, the obtained results revealed that the high incidence was in winter with an incidence of 42.4% more than in autumn, spring and summer as they recorded an

incidence of 9%, 19.8%, and 13.6% respectively. According to Kandeil et al. (2019), who stated that H5N8 positive samples were mostly detected in colder weather. This cold weather favours the survival of the virus, in addition to the high ammonia levels with bad ventilation in multiple commercial farms, causing further complications and increase the infection risk (Seififi et al., 2010). Additionally, samples were collected from different types of ducks; Sudani, Pekin, and Muscovy. Consequently, we detected that Sudani ducks were highly susceptible to infection with HPAI H5N8 with a ratio of 71.2% while other types Pekin and Muscovy recorded infection ratios of 21.2% and 7.57%, respectively. On the other hand, Sultan et al. (2019) reported that Muscovy ducks seem to be more liable to clade 2.3.4.4b HPAI H5N8 infection compared to other breeds, and this is may be due to the innate immunological difference of ducks or their adaptation level, and the difference in the antibody levels associated with vaccination, in addition to the antigenic evolution and adaptability of the H5N1 and H5N8 viruses (Cagle et al., 2012; Pantin & Suarez 2013). In the current study, number of samples collected from Sudani ducks was 47 in comparison to Pekin and Muscovy ducks which were 14 and 5 respectively, and this was attributes to marketing purposes. RT-qPCR is a confirmed molecular technique used worldwide as a diagnostic tool for most poultry viral diseases including the H5 Avian influenza virus (Ghafouri et al., 2017). All H5Nx strains detected in ducks were characterized as HPAI H5N8. In the present study, one sample was selected upon the year examined, mortality rate, and positive results of RT- qPCR with low Ct value, seasonal and geographical distribution. The selected sample was from Giza governorate and collected in 2020, and for molecular characterization, the selected sample was isolated and propagated in specific pathogen-free Eggs (SPF ECEs) as AIV was completely adapted in 9-11 day old SPF ECE via allantoic sac inoculation route (Sedeik et al., 2018). In the isolated sample (A/Duck/Egypt/SA1/2020H5N8), amino acid sequences of the HA cleavage site revealed polybasic amino acid which is characteristic of HPAIV, clade 2.3.4.4. The amino acid sequences of HA cleavage of H5N8 isolates were (PLREKRRKR/GLF), are similar to those previously reported by (Kandeil et al., 2017; Anis et al., 2018; Shehata et al., 2019; Hassan et al., 2020).

To examine the correlation between the genetic distances of the currently circulating HPAI H5N8, we performed a phylogenetic relationship between the isolated sample A/Duck/Egypt/SA1/2020(H5N8) and other AIV isolates based on the HA gene nucleotide sequence. The HPAI H5N8 isolate is clustered in clade 2.3.4.4b with HA amino acid identity percent ranging from (89.6% - to 92.6%) with the Egyptian isolates, in agreement with Hamouda et al. (2019); Shehata et al. (2019). H5N8 isolate shows a similarity between the other H5N8 isolates, from Asia (Eastern China, Korea) and from Europe (Denmark, England and France) during 2016-to 2020 with HA amino acid identity percent ranging from (95.5% - to 96.6%), and Russia (Moscow) with amino acid identity percent with each other (96.1%). Our result is analogous to those Kandeil et al. (2017); Selim et al. (2017) & Yehia et al. (2018). The amino acid identity percent between the local vaccinal strains and the field isolates was ranging from (95.8% - to 96.4%). While H5N1 vaccinal strain showed relativity low distinct range ranging from (88.6% - to 92.6%), which agreed with the results of Kandeil et al. (2017).

The pathological evaluation of infected ducks revealed gross lesions that was mainly restricted to congestion and hemorrhage involving different organs including lung, heat, liver, pancreas, spleen, kidney and brain. The histopathological alterations were mainly necrotic and acute inflammatory in nature in addition to vascular reaction associated with edema and hemorrhage. These reactions were attributed to direct viral replication in these tissues as confirmed by immunohistochemistry. Necrotic and inflammatory reactions induced by virus indicated the systemic dissemination of the virus in the infected ducks resulting in of death in infected birds. The present data confirmed that H5N8 is highly pathogenic in ducks while mortalities is related to the high cleavability of heamagglutinin (HA) facilitate the virus entry through the vasculature as discussed by Feldmann et al. (2000). Moreover, Christine et al. (2008) reported that direct viral replication and dysregulation of cytokines and chemokines in addition to upregulation of tumor necrosis factor related apoptosis inducing ligands were involved in the pathogenesis of virulent strains of avian influenza. The present study showed that the isolated strain of AIV is highly pathogenic in domestic ducks as reflected by severe developed pathological alterations involving different tissues. Gaide et al. (2022) stated that ducks developed relatively mild viral RNA load compared with chicken. On contrary, Chicken developed severe lesions in different organs after experimental infection by HPAI H5N8 that are able to replicate systemically in all organs (Yehia et al., 2022).

The respiratory lesions included tracheal deciliation, congestion and mild inflammatory reaction is related to residence of viral antigen in tracheal epithelium as detected by expression of viral antigen in tracheal epithelium. Pulmonary lesions were restricted to severe vascular with mild inflammatory reactions similar findings was described by Brojer et al. (2009). The present data revealed that the main lesions in the heart were subepicardial edema with intermyocardial edema and hemorrhages associated with necrosis of myofibers accompanied by few lymphohistiocytic infiltrations, The pathological picture is related to direct

viral replication in cardiac myofibers as confirmed by immunolabelling of viral antigen Previous investigation confirmed the ability of HPAI to cardiac myocytes (Swayne et al., 2013; Núñez et al., 2016; Punnoose et al., 2021). In addition, Abdo et al., 2014 observed viral antigens in necrotic cardiac myocytes by immunolabelling. Microscopic examination of liver sections from the affected cases revealed necroapoptotic reaction involving hepatocytes with sinusoidal lymphocytosis and severe congestion. These lesions are related to residence of viral antigen in vascular endothelium and in hepatocellular cytoplasm as confirmed by immunostaining as described by Brojer et al. (2009) and Abou-Rawash et al. (2012). Microscopically, the pancreas showed necrotic pancreatitis characterized by vacuolations and multifocal necrosis with lymphocytic cell infiltration in pancreatic acini which is similar to Núñez et al. (2016) who reported that pancreatic necrosis was seen in in ducks. Outbreaks Studies given by Abdo et al. (2014) and Mohammed et al. (2018) confirmed the viral residence in necrotic pancreatic acini.

Severe necrotic reaction with hemorrhage were detected in duodenum with presence of strong expression of viral antigen in duodenal crypts, this residence of viral antigen in intestinal crypt may be related to villous atrophy and profound diarrhea. AIVs isolated from domestic ducks can replicate in the intestinal tract and this was attributed to their ability to maintain sialidase activities under lower pH conditions (Takahashi et al., 2001; Evseev & Magor, 2019).

In the current study, the spleen in the affected ducks exhibited necrotic splenitis with severe congestion and fibrinoid necrosis of splenic arterioles associated with lymphoid depletions. The immunohistochemical localization of virus in splenic endothelium is related to developed necrotic reaction as reported by Abou-Rawash et al. (2012). Congestion of peritubular capillaries with mild degeneration of renal tubules, and discrete foci of tubular epithelial necrosis were detected in the kidney of the affected ducks, the renal lesions may be attributed to systemic inflammatory cytokines expression (Kwon et al., 2005; Abou-Rawash et al., 2012).

Brain of ducks infected by AIV H5N8 showed acute inflammatory lesions including nonsuppurative encephalitis characterized by gliosis, brain edema, and congestion, explaining the nervous manifestation in the affected birds. Similar lesions were reported in subtypes H5N2-, H5N6- and H5N8-infected domestic ducks (Banyai et al., 2016). In addition to the presence of microthrombosis, cerebral lesions could be claimed to direct viral replication in capillary endothelium resulting in endothelial swelling, injury and subsequent thrombosis, vasculitis and related malacia as confirmed by immunohistochemistry. The observed lesions indicated a high neuro-and endothelio viral tropism. The residence of viral antigen in capillary endothelium, neurons and glia cells was confirmed by immunohistochemistry as described by Mohammed et al. (2018). The neuro invasive nature of the H5N8 strain was previously reported by Banyai et al. (2016), who found in the brains of inoculated ducks with H5N6 and H5N8 higher viral titers compared with those of the H5N2 infected ones.

CONCLUSION

The present study showed great susceptibility of domestic ducks to natural infection by HPAI subtype H5N8, with seasonal and breed difference. The wide dissemination of virus in various organs and localization in specific cells were related to developed histopathological lesions in theses tissues, and this confirm the pantropic nature of the virus in ducks.

ACKNOWLEDGEMENTS

We would like to thank all colleagues and coworkers in the Pathology department, Faculty of Veterinary Medicine, Cairo University, Egypt, for their technical support.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interests.

NOVELTY STATEMENT

This paper is focused on the recent outbreaks occurred in duck farms that were infected with H5N8 Influenza in Egypt and representing antigenic correlation between the virus residence and developed pathology in different tissue of infected ducks.

AUTHORS CONTRIBUTION

Sara.M.H, Formal Analysis and investigation,paper, Writing– original draft preparation writing, Faten fathy: Microscopic analysis Writing– review and editing, Elshaimaa Ismael: Propation of virus in ECH, HA and HI tests, Mohamed Tarek: Perform phylogenetic analysis. Fatma Abdel Reheem: Perform rPCR, Rawhia Esawy Doghaim: Conceptualization and supervision and revision.

REFERENCES

Abdelwhab EM, Hafez HM (2011). An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: epidemiology and control challenges. Epidemiol. Infect. 139: 647–657. https://doi.org/10.1017/S0950268810003122.

Abdo w, Haridy m, Katou Y, Goto M, Mizoguchi T, Sakoda Y, Yanai T (2014). Pathological and Immunohistochemical Findings of Natural Highly Pathogenic Avian Influenza Infection in Tufted Ducks during 2010–2011 Outbreaks in Japan. J. Vet. Med. Sci., 76(9): 1285–1290. http://doi.org/10.1292/jvms.14-0084.

Abou-Rawash AA, EL-Hamed HS, Abd-Ellatieff HA, Elsamanoudy SM (2012). Recent Outbreaks of Highly Pathogenic Avian Influenza Virus in Chickens and Ducks in Egypt: Pathological Study.

Adlhoch C, Gossner C, Koch G, Brown I, Bouwstra R, Verdonck F, Penttinen P, Harder T, (2014). Comparing introduction to Europe of highly pathogenic avian influenza viruses A(H5N8) in 2014 and A(H5N1) in 2005. Euro Surveill 19: 20996.

Alexander DJ (2007). An overview of the epidemiology of avian influenza. Vaccine., 25 (30):5637-5644.

Alexander J.D, Brown IH (2009). History of highly pathogenic avian influenza, Rev. Sci. Tech. 28 19–38. https://doi.org/10.20506/rst.28.1.1856.

Aly MM, Arafa A, Hassan MK (2008). Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. Avian Dis. 52, 269–277. https://doi.org/10.1637/8166-103007-Reg.1.

Anis AM, AboElkhair M, Ibrahim (2018). Characterization of highly pathogenic avian influenza H5N8 virus from Egyptian domestic waterfowl in 2017. Avian Pathol., 47 (4).

Bancroft J.D., Layton C. (2013). The hematoxylins and eosin. In: Suvarna, S. K., Layton, C. and Bancroft, J.D. (eds) Bancroft's Theory and Practice of Histological Techniques, 7th ed. Churchill Livingstone, London, UK. pp. 172–186.

Banyai K, Bistyak AT, Thuma A, Gyuris E, Ursu K, Marto S, Farkas L, Hortobágyi E, Bacsadi Á, Dan A (2016). Neuroinvasive influenza virus A (H5N8) in fattening ducks, Hungary, 2015. Infect., Genet. Evol., 43: 418–423.

Barber MR, Aldridge Jr JR, Webster RG, Magor KE (2010). Association of RIG-I with innate immunity of ducks to influenza. Proc. Natl. Acad. Sci. USA;107(13):5913– 5918.

Bernard N Fields, David M Knipe, Peter M Howley (2013). Fields Virology 6th Edition,. Wolters Kluwer Health/ Lippincott Williams & Wilkins, ©2013.Philadelphia, pp 1151–1185.

Bröjer C, Agren EO, Uhlhorn H, Bernodt K, Morner T, Jansson DS, Mattsson R, Zohari S, Thoren P, Berg M, Gavier-Widen D (2009). Pathology of natural highly pathogenic avian influenza H5N1 infection in wild tufted ducks (*Aythya fuligula*). J. Vet. Diagn. Invest. 21: 579–587. http://doi.org/10.1177/104063870902100501.

Cagle C, Wasilenko J, Adams SC, Cardona CJ, To TL, Nguyen T, Spackman E, Suarez, DL, Smith D (2012). Differences in Pathogenicity, Response to Vaccination, and Innate Immune Responses in Different Types of Ducks Infected with a Virulent H5N1 Highly Pathogenic Avian Influenza Virus from Vietnam. Avian Dis. 56: 479–487.

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

Christine Korteweg MD, Jiang Gu (2008). Pathology, Molecular Biology, and Pathogenesis of Avian Influenza A (H5N1) Infection in Human. Am. J. Pathol. 172(5): 1155–1170. http://doi.org/10.2353/ajpath.2008.070791.

CIDRAP, Centre for Infectious Disease Research and Policy (2016). Avian Flu in Germany and France. Available from: http://www.cidrap.umn.edu/news-perspective/2016/12/more-avian-flu-reported-germany-france.

Cox NJ, Fuller F, Kaverin N, Klenk HD, Lamb RA, Mahy BW, McCauley JW, Nakamura K, Palese P, Webster RG (2000). Orthomyxoviridae. In: Virus taxonomy. Seventh report of the international committee on taxonomy of viruses, M.H. Van Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B.Carstens, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle, and R.B. Wickner, eds. Academic Press,San Deigo. 585–597.

Evseev D , Magor K (2019). Innate Immune Responses to Avian Influenza Viruses in Ducks and Chickens. Vet. Sci., 6(1): 5.

Feldmann A, Schäfer MK, Garten W, Klenk HD (2000). Targeted infection of endothelial cells by avian influenza virus A/FPV/Rostock/34 (H7N1) in chicken embryos. J. Virol. 2000 Sep;74(17):8018-27. http://doi.org/10.1128/jvi.74.17.8018-8027.2000. PMID: 10933711; PMCID: PMC112334.

Gaide N, Lucas M, Delpont M, Croville G, Bouwman K, Papanikolaou A, van der Woude R, Gagarinov I, Boons G, De Vries R, Volmer R, Teillaud A, Vergne T, Bleuart C, Le Loc'h G, Delverdier M, Guérin J (2022). Pathobiology of highly pathogenic H5 avian influenza viruses in naturally infected Galliformes and Anseriformes in France during winter 2015–2016. Vet. Res., 53(1).

Ghafouri SA, Ghalyanchi Langeroudi AH, Maghsoudloo, Farahani RKH, Abdollahi H., Tehrani1 F, Fallah MH (2017). Clade 2.3.4.4 avian influenza A (H5N8) outbreak in commercial poultry, Iran, 2016: the first report and update.

Hall T Bio (1999). A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. NucleicAcids Symp. Ser., 41: 95–98.

Hamouda Amira H, Awad Abd El-hafez, Mahmoud G Ibrahim, Hesham A sultan (2019). Genetic and Antigenic Relatedness of Highly Pathogenic Avian Influenza H5Nx Viruses Currently Circulating in Egypt and Some H5 Commercial Vaccines. J. Curr. Vet. Res., 1(1): 60-74. http://doi.org/10.21608/jcvr.2019.10168.1000.

Hassan K E, Ali A, Dahshan AHM, El-Sawah AA, Shany SAS, El-Kady MF (2016). Prevalence of avian respiratory viruses in broiler flocks in Egypt. Poult. Sci., 95(6): 1271–1280. https ://doi. org/10.3382/ps/pew068.

Hassan KE, Noha Saad, Hassanein H Abozeid, Salama Shany, Magdy F El-Kady, Abdelsatar Arafa, Azza A A El-Sawah, Florian Pfaff, Hafez M Hafez, Martin Beer, Timm Harder (2020). Genotyping and reassortment analysis of highly pathogenic avian influenza viruses H5N8 and H5N2 from Egypt reveals successive annual replacement of genotypes. Infection, Genet. Evol., 84, p. 104375. http://doi.org/10.1016/j.meegid.2020.104375.

Hill SC, Lee YJ, Song BM, Kan HM, Lee EK, Hanna A, Gilbert M, Brown I H , Pybus OG (2015). Wild waterfowl migration and domestic duck density shape the epidemiology of highly pathogenic H5N8 influenza in the Republic of Korea. Infect., Genet. Evol., 34: 267–277.

Hoffmann B, Hoffmann D, Henritzi D, Beer M, Harder TC (2016). Riems influenza a typing array (RITA): an RT-qPCR-based low-density array for subtyping avian and mammalian influenza a viruses. Sci. Rep. 6: 27211.

Hoper D, Hoffmann B, Beer M (2009). Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes. J. Clin. Microbiol., 47: 674–679.

Horwitz MS, Scharff MD (1969). The production of antisera

against viral antigens. In Habel, K and Salzman, N.P.Fundamen. Techniq. Virol. Pp 223-262.

Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepangestie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature. 8;430(6996):209-13. doi: 10.1038/nature02746. PMID: 15241415.

Kandeil A, Kayed A, Moatasim Y, Webby RJ, McKenzie PP, Kayali G, Ali MA (2017). Genetic characterization of highly patho- genic avian influenza A H5N8 viruses isolated from wild birds in Egypt. J. Gen. Virol., 98(7): 1573–1586. https://doi. org/10.1099/jgv.0.000847.

Kandeil Ahmed, Hicks Joseph T, Young, Sean G, El Taweel Ahmed N, Kayed Ahmed S, Moatasim Yassmin, Kutka Omnia, Bagato Ola, McKenzie Pamela P, Cai Zhipen, Badra Rebecca, Kutkat Mohamed, Bahl Justin, Webby Richard J, Kayali Ghazi, Ali Mohamed A (2019). Active surveillance and genetic evolution of avian influenza viruses in Egypt. 2016–2018. Emerg. Microb. Infect., 8(1): 1370–1382. http://doi.org/10.1080/22221751.2019.1663712.

Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol., 30: 772–780.

Krauss S, Obert CA, Franks J, Walker D, Jones K, Seiler P (2007). Influenza in migratory birds and evidence of limited intercontinental virus exchange. Plos Pathog.;3:e167.

Kwon YK, Joh SJ, Kim MC (2005). Highly pathogenic avian influenza in magpies (Pica pica sericea) in South Korea. J. Wildl. Dis. 41:618–623.

McBride DS, Lauterbach SE, Li YT, Smith GJD, Killian ML, Nolting JM, Su YCF, Bowman AS (2021). Genomic Evidence for Sequestration of Influenza A Virus Lineages in Sea Duck Host Species. Viruses. 13: 172. https://doi.org/10.3390/v13020172.

Mohammad QA, Mahmoud NA (2005).Sero-prevalence of avian influenza among broiler-breeder flocks in Jordan. Prevent. Vet. Med. 70 : 45-50.

Mohammed Nooruzzaman, Md. Enamul Haque, Emdadul Haque Chowdhury, Mohammad Rafiqul Islam (2018). Pathology of Clade 2.3.2.1 Avian Influenza Virus (H5N1) Infection in Quails and Ducks in Bangladesh. Avian Pathol., http://doi.org/10.1080/03079457.2018.1535165.

Naguib MM, El-Kady MF, Lüschow D, Hassan KE, Arafa AS, El-Zanaty A, Hassan MK, Hafez HM, Grund C, Harder TC (2017). New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle Disease viruses. J. Virol. Methods., 245: 19–27.

Neumann G (2015). H5N1 influenza virulence, pathogenicity and transmissibility: what do we know? Future Virol.; 10(8):971-980. http://doi.org/10.2217/fvl.15.62. PMID: 26617665; PMCID: PMC4658657.

Núñez A, Brookes SM, Reid SM, Garcia-Rueda C, Hicks DJ, Seekings JM, Spencer YI, Brown IH (2016). Highly pathogenic avian influenza H5N8 clade 2.3.4.4 virus: equivocal pathogenicity and implications for surveillance following natural infection in breeder ducks in the United Kingdom. Transbound Emerg. Dis. 63: 5-9.

Pantin-Jackwood M J, Suarez DL (2013). Vaccination of domestic ducks against H5N1 HPAI: a review. Virus Res. 178:21–34.

Parvin R, Schinkoethe J, Grund C, Ulrich R, Bönte F, Behr KP ,Voss, M, Samad MA (2020). Hassan, K.E.; Luttermann, C.; et al. Comparison of pathogenicity of subtype H9 avian influenza wild-type viruses from a wide geographic origin expressing mono-, di-, or tri-basic hemagglutinin cleavage sites. Vet. Res. 51: 48.

Pohlmann A, Staric E, Harder T, Grund C, Hoper D, Globig A, Staubach C, Dietze K, Strebelow G, Ulrich RG, Schinkothe J, Teifke JP, Conraths FJ, Mettenleiter TC, Beer M (2017). Outbreaks among wild birds and domestic poultry caused by reassorted influenza A(H5N8) clade 2.3.4.4 viruses, Germany, 2016. Emerg. Infect. Dis. 23: 633–636.

Punnoose P, Vineetha S , Mahesh M (2021).Current scenario and pathology of duck diseases - A systematic review',. *Indian J. Vet. Pathol.*, 45(4): 242–265. http://doi.org/10.5958/0973-970x.2021.00046.8.

Ruba T, Siddiqua MT, Islam MS, Mithun RP, Hossain MM, Ali MA, Khan (2015). Pathology, pathotype and phylogenetic analyses of a natural outbreak of highly pathogenic avian influenza (HPAI) virus in farming ducks of Bangladesh. IOSR J. Agric. Vet. Sci., 8 (10) Ver. II , 137-146. http://doi.org/10.9790/2380-08102137146.

Sedeik M El-S, Elshal NA, Awad AM and Kandil N (2018). Molecular Survey and Characterization of H5N8 Isolates during 2016-2017 on Egypt. J. World Poult. Res., 8 (4): 127-133. http://jwpr.science-line.com.

Seififi S, Asasi K , Mohammadi A (2010) .Natural co-infection caused by avian inflfl uenza H9 subtype and Infectious bronchitis viruses in broiler chicken farms. Vet. Arhiv. 80: 269-81.

Selim AA, Erfan AM, Hagag N, Zanaty A, Samir AH, Samy M, Abdelhalim A, Arafa ASA, Soliman MA, Shaheen M, Ibraheem EM, Mahrous I, Hassan MK, Naguib MM (2017). Highly pathogenic avian influenza virus (H5N8) clade 2.3.4.4 infection in migratory birds, Egypt. Emerg. Infect. Dis. 23: 1048–1051. https://doi.org/10.3201/eid2306.162056.

Shehata AA, Seddik M, Elbestawy A, ZainEl-Abideen MA, Hammam H, Kilany WK, Ali A (2019). Co- infection, genetic, and antigenic relatedness of Avian Influenza H5N8 and H5N1 viruses in domestic and wild birds in Egypt. Poult. Sci. 0: 1-9.

Simpura J (1998). Mediterranean mysteries: Mechanisms of declining alcohol consumption. Addiction., 93(9): 1301–1304.

Smith GJ, Donis RO,World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5 Evolution Working Group (2015). Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. Influenza Other Respir Viruses. 2015 Sep;9(5):271-6. http://doi.org/10.1111/irv.12324.

Sonnberg S, Webby RJ, Webster RG (2013). Natural history of highly pathogenic avian influenza H5N1. Virus Res.; 178:63–77.

Sultan Hesham A., Abd-Elsatar Arafa, Shaimaa Talaat, Alaa A. Gaballa, Walid H. Kilany, Wael K. Elfeil, Awad A. Shehata, and N. Amarin (2019). Efficacy of Clade 2.3.2 H5-Recombinant Baculovirus Vaccine in Protecting Muscovy and Pekin Ducks from Clade 2.3.4.4 H5N8 Highly Pathogenic Avian Influenza Infection. AVIAN DIS.

63:219–229, 2019.

Swayne DE, Suarez DL, Sims LD (2013). Influenza. In: Diseases of Poultry/ D.E. Swayne, J.R. Glisson, L.R. McDougald, V. Nair, L.K. Nolan, and D.L. Suarez, eds. Wiley-Blackwell, Ames, Iowa.181–218.

Takahashi T. Suzuki Y, Nishinaka D. Kawase N. Kobayashi Y, Hindari KIPJ., Miyamoto D, Guo CT, Shortridge KF, Suzuki T (2001). Duck and human pandemic infl uenza A viruses retain sialidase activity under low pH conditions. J. Biochem. 130: 279-283.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Molecul. Biol. Evol. 30: 2725-2729.

Van Wijhe M, Ingholt MM, Andreasen V, Simonsen L (2018). Loose ends in the epidemiology of the 1918 Pandemic: Explaining the extreme mortality risk in young adults. Am. J. Epidemiol. 187: 2503–2510. https://doi.org/10.1093/aje/kwy148 PMID: 30192906.

Xu X , Subbarao , Cox NJ, GuoY (1999).Genetic characterization of the pathogenic influenza A/Goose/ Guangdong/1/96 (H5N1) virus: Similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology. 261:15–9.

Yehia N, Naguib MM, Li R, Hagag N, El-Husseiny M, Mosaad Z, Nour A, Rabea N, Hasan WM, Hassan MK, Harder T, Arafa AA (2018). Multiple introductions of reassorted highly pathogenic avian influenza viruses (H5N8) clade 2.3.4.4b causing outbreaks in wild birds and poultry in Egypt. Infection, Genet. Evol., 58: 56–65

Yehia N, Erfan AM, Adel A, El-Tayeb A, Hassan WMM, Samy A, Abd El-Hack ME, El-Saadony MT, El-Tarabily KA, Ahmed KA (2022). Pathogenicity of three genetically distinct and highly pathogenic Egyptian H5N8 avian influenza viruses in chickens. Poult. Sci. 2022 Mar;101(3):101662. http://doi.org/10.1016/j.psj.2021.101662.