Research Article

Genetic Characterization of Highly Pathogenic Avian Influenza Virus (H5N8) in Backyard Poultry Production Sector during Mid-2017 to Autumn 2018

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Abstract | Since incursion of highly pathogenic avian influenza virus (HPAIV) H5N8 in November 2016 in Egypt, it caused severe economic losses for both the commercial and backyard poultry production sectors. The aim of this work is to study the Situation and molecular characterization of HPAIV (H5N8) in backyard poultry production sector in Egypt. In this study, a total of 7505 samples of tracheal swabs representing 1180 backyard poultry flocks were collected based on the surveillance conducted by the GOVS (General Organization for Veterinary Services), Egypt during the period from May 2017 to August 2018. 11 positive cases were confirmed in seven governorates with a prevalence rate of 0.93% and the positive samples were mainly located in Upper Egypt. The sequence of partial HA gene was performed for 10 positive samples and they were genetically characterized as HPAIV H5N8 belongs to clade 2.3.4.4b. Except three isolates in 2018 belongs to clade 2.3.4.4A, the phylogenetic analysis revealed that our sequenced viruses were clustered together in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4.

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Introduction

The HPAI (H5N8) AI virus was first detected during 2010 in breeding ducks in China, it belonged to clade 2.3.4.4 [19, 32]. In 2014, the HPAI (H5N8) was detected in wild and domestic bird in South Korea then spread to Europe, North America, and East Asia via migratory birds [8, 13, 27].

Two distinct clusters of HPAI (H5N8) viruses have been identified: group A viruses (Buan-like: A/Broiler-duck/Korea/Buan2/2014) and group B [A/breeder-duck/Korea/Gochang1/2014, Gochang-like] [20]. Egypt reported their first detection of the H5N8 HPAI virus in November 2016 from aquatic wild birds (common coot and green-winged teal) [18, 29] which was closely related to European HPAI H5N8 viruses of clade 2.3.4.4b. Full genome sequencing of the virus revealed three different reassortant HPAI H5N8 viruses were detected in Egypt among wild and domestic birds [37]. Egypt has a large number of backyard flock reach 8.1 million households all over

the Egyptian country [3, 21]. Flock size can range from 10–20 birds up to a few hundred [16].

The backyard poultry production is an important source of meat and eggs in Egypt, it gives about 53% of daily needs from protein compared to bovine and sheep meat [12]. In addition, it constitutes about 10% of the market meat production sector and 30% of the egg market. It is usually composed of mixed bird species that support the source of integration of new viruses from wild birds to the backyard sector and so helps the spread of viruses to other nearby commercial farms. This sector is usually based on poor biosecurity and lack of veterinary observation or vaccination [17, 2]. So, the backyard poultry has become a threat to the commercial poultry industry due to it is a constant reservoir for avian influenza virus (AIV) [35, 6].

HA is the major surface glycoprotein of AIV. It mediates binding of the virion to host cell receptors and fusion between the virion envelope and endosomal membranes. The HA protein appears to be the most important protein in determining the virulence of AI viruses. For the virus to become infectious, cleavage of a precursor of HA [HA0] into HA1 and HA2 subunits (linked by a single disulfide bond) is required, since cell fusion is mediated by the free amino-terminus of the HA2 subunit [9].

The most important determinant of pathogenicity is the cleavage site structure of the HA. Analysis of the HA1-HA2 junction regions in influenza viruses with different pathogenicity revealed the presence of a stretch of basic residues in the HA of pathogenic strains [7].

Poultry industry infrastructure

In general, poultry production in Egypt, as mentioned earlier, is divided into four sectors based on implementation of biosecurity measures; however, many farms in sectors 3 and 4 are not registered with the official authorities which hinders the monitoring and early recognition of infections and allows silent and wide spread of the virus. Reforming of the poultry industry infrastructure in Egypt is a fundamental approach to the control of HPAI.

Backyard birds

Although the majority of householders keep mainly ducks and chickens together, nevertheless rearing of geese, turkeys and pigeons in close contact with other animals and humans in the same household is a common practice in Egypt [4]. Some years ago, the government encouraged this production sector by small loans and marketing facilities. Up to the end of the 1970s, rural poultry production was an important source of Egypt's poultry meat and eggs. Rural poultry production prior to the HPAI crisis was esti-mated to be about 10 % of the market share of the meat production sector and 30 % of the egg market. Backyard birds produced 22 % of chicken meat, 64% of ducks, 34% of turkeys, and almost all geese and pigeons [39]. Flock size can range from 10–20 birds up to a few hundred [2]. It is estimated that backyard birds are mostly reared in primitive cages, rooftops, or as scavengers with virtually no biosecurity [Fig. 2]. They move or graze through streets, roads or fields. These birds are in close contact with either local feral birds and/or wild migratory birds [4, 5]. The attitude of the backyard birds' householders hinders cooperation with vaccination committees. In some cases, they refuse the vaccine and hide their birds without vaccination or they may vaccinate some birds and leave others unvaccinated. Moreover, backyard waterfowl in Egypt are considered a potential reservoir of the virus and a mixing vessel for selection of variants to infect humans [20] or break through the immune system, and cause infection in vaccinated birds [8]. But under village conditions it is not practical to separate the different species and such a suggestion will complicate the control efforts [7].

The current work aims to study the situation, epidemiological mapping and genetic characterization of HPAI (H5N8) in backyard based on surveillance conducted by GOVs and the National laboratory for veterinary quality control on poultry production (NLQP) in Egypt during the period from May 2017 to August 2018, as well as sequence of partial HA gene of H5N8 AI including cleavage site was carried out from backyard sector in different governorates.

Materials and Methods

Sampling

A total of 7505 different samples of tracheal swabs that represented 1180 suspected flocks were collected from suspected avian influenza infected poultry backyard.

Detection of AIV H5 subtype by real-time RT-PCR

The viral RNA extraction from the pooled swabs

(10 swabs/ pool) was performed using a Qia Amp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturing instructions. All the samples were tested for the M gene of influenza type A by One Step Real-Time RT-PCR Kit (QIAGEN, Hilden, Germany) using specific sets of primers and probes for M gene [34].

Then Positive AIV samples were subtyped for HA and NA using specific subtyping RT-qPCR [15, 22].

Virus isolation

The positive samples were propagated in SPF ECE. 0.2 ml of each sample was inoculated in five specific pathogen free embryonated chicken eggs (SPF ECE) of 9-11 days old via allantoic sac with daily candling up to 3 days for embryonic deaths and after 48 and 72 hours the allantoic fluid was harvested for confirmation by HA test.

Haemagglutination (HA) test

Plate HA test was done on the isolates for confirmation of the H5 subtype of avian influenza virus using standard protocol. Dispense the 25 µl of PBS into each well and then 25 µl of virus suspension [i.e. infective allantoic fluid] then two-fold dilutions of 25 µl volumes of the virus suspension were done across the plate. Then 25 µl of 1% (v/v) chicken RBCs was dispensed to each well. Mixing was done by tapping the plate gently and then the RBCs were allowed to settle for about 40 minutes at room temperature. HA was determined by observing the presence or absence of tear-shaped streaming of the RBCs [25].

Genotyping of H5N8 AIV virus

The positive isolates for HPAI AIV (H5N8) were sequenced to detect the genotype of HPAI. The viral RNA was extracted from the isolated samples and amplified by using primers specific for partial HA gene including cleavage site KH15-CCTCCAGARTATGCMTAYAAAATTGTC-3, KH3 5TACCAACCGTCTACCATKCCY-TG-3 using one-step RT-PCR with Qiagen®kit (QIAGEN, Hilden, Germany) [23] according to manual instruction. Gel purification of the positive samples was done by using Qiagen gel extraction kit (QIAGEN, Hilden, Germany).

Sequencing of partial HA gene

The positive PCR products were sequenced for partial HA gene including cleavage site using Big Dye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) according to manual instruction with Genetic analyzer (ABI-3130, Applied Biosystems, Foster City, CA), using HA gene cleavage site specific primers [23].

Genetic and phylogenetic analysis

Sequence alignments of Nucleotide and amino acid sequence were performed in comparison with other related strains obtained from GeneBank and GISAID database using MegAlign module of DNASTAR software [Lasergene version 7.2; DNASTAR, Madison, WI, USA] using the CLUSTAL-W program. The phylogenetic tree was generated using MEGA version 6 [www.megasoftware.net] by neighbor-joining [N–J] tree method. The pair-wise nucleotide percent identity was calculated using DNA star software (Lasergene version 7.2; DNASTAR, Madison, WI, USA).

Results and Discussion

Detection of AIV H5N8 subtype by real-time RT-PCR

From 1 May 2017 to 31 August 2018, only 11 HPAI H5N8 samples were positive by real-time PCR for H5 and N8 genes in seven governorates, with a prevalence rate of 0.93% (Figure 1). The H5N8 cases were recorded in following 7 governorates [Al Giza, El Wadi El Gedeed, Qena, Sohag, El Moneifia, Suez and Minia] from total 22 investigated governorates, it founds in chickens (5), and ducks (6), and the recorded geo-prevalence of 31.8 %.

The positive cases were mainly located in upper Egypt than lower Egypt (Table 1), with 1.6% [6 of 374] distributed in El Wadi El Gedeed (1), Qena (2), AL-Minia (1), Sohag (2) (Figure 2).

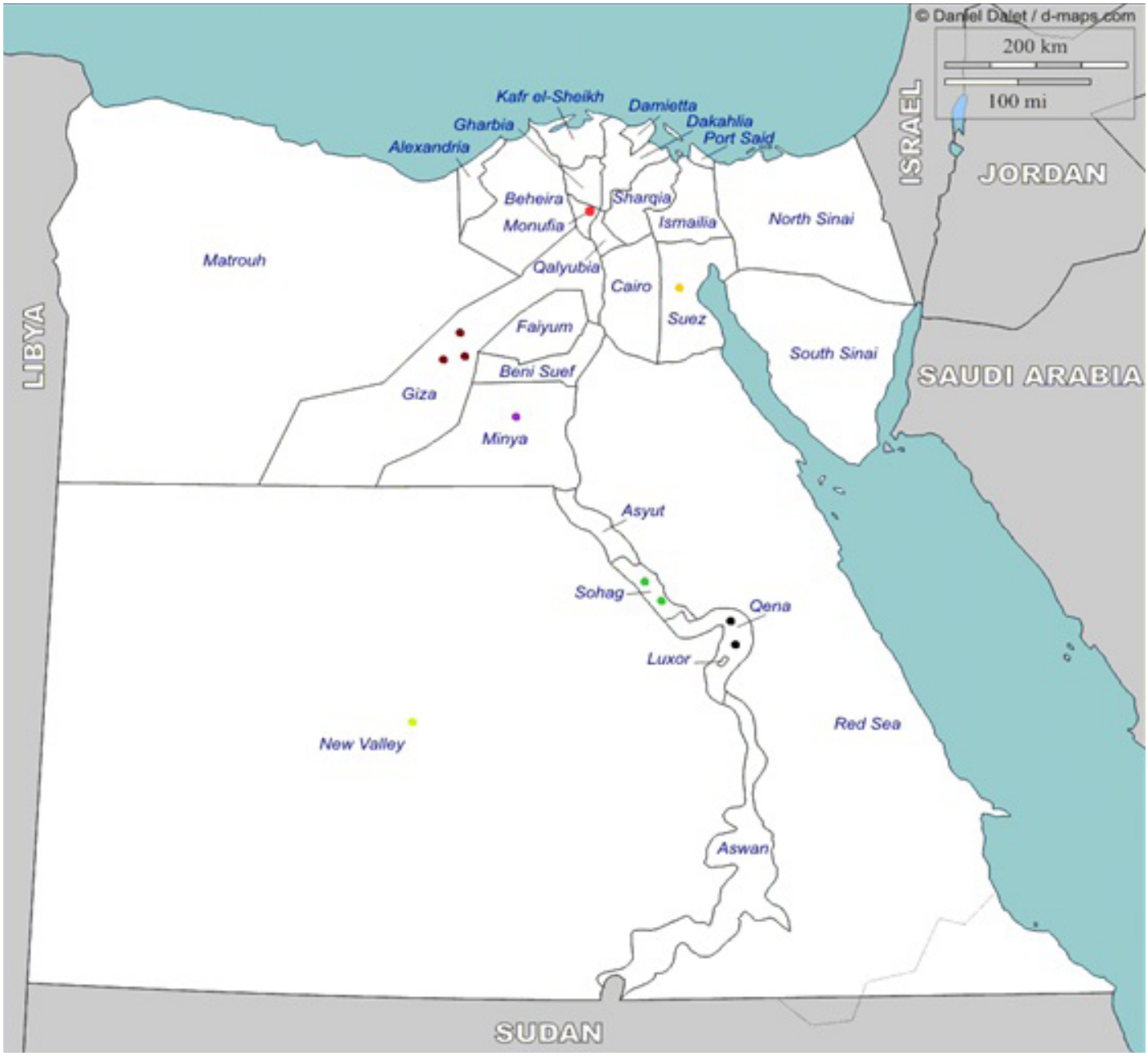

Figure 1: *Geographical distribution of HPAI H5N8 during May 2017-August 2018 in Egypt.* **FN:** *The Fig. show the distribution of the HPAI H5N8 positive cases mainly in upper Egypt.*

Table 1: *Percent of positive cases examined from different regions during May 2017-August 2018 in Egypt.*

Examined	Positive cases	%	Region
374	6	1.6%	Upper
623	4	0.6%	Lower
145	1	0.7%	East
38	0	0.0%	West
1180	11	0.93	Total

The table shows the highest percent in upper Egypt than in other regions. Upper Egypt (Al Menia, Sohag, Qena, Al-Wady Al-Gadid), Lower Egypt (Giza, Al Menofia), East Egypt (Suez).

Virus isolation and HA test

Ten positive samples were positive after isolation by HA. Only one sample failed in isolation and give negative results by HA after isolation.

Sequencing and phylogenetic analysis

The Phylogenetic analysis of partial HA gene including the cleavage site of 10 sequenced viruses were clustered in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4. (Figure 3). The cleavage site of seven samples resembled AI H5N8 clade 2.3.4.4 Group B (PLREKRRKRGLF) and the other three samples from 2018 resembled Group A (PLRERRRKRGLF) (Table 2).

Comparative alignment of partial HA gene showed that HA genes of the 10 viruses share a similarity

Table 2: *Amino acid residues analysis of the HA gene cleavage site of the sequenced HPAI H5N8 viruses among different proteins.*

Clade	Cleavage site	Virus ID
2.3.4.4B	PLREKRRKRGLF	A/chicken/Czech_Republic/206-17_2/2017
	PLREKRRKRGLF	A-tufted-duck-Germany-AR8444-2016
	PLREKRRKRGLF	A-tufted-duck-Germany-AR8459-2016
	PLREKRRKRGLF	A-mallard-duck-Korea-WA137-2017
	PLREKRRKRGLF	A-breeder-duck-Korea-Gochang1-2014
	PLREKRRKRGLF	A-duck-Zhejiang-6D18-2013
	PLREKRRKRGLF	A-goose-Shandong-WFSG1-2014
	PLREKRRKRGLF	A-Duck-Egypt-17167S-2017
	PRREKRRKRGLF	A-Duck-Egypt-1727-2017
	PLREKRRKRGLF	A-Chicken-Egypt-1836CAL-2018
	PLREKRRKRGLF	A-Chicken-Egypt-1735CA-2017
	PLREKRRKRGLF	A-Duck-Egypt-1757SM-2017
	PLREKRRKRGLF	A-Duck-Egypt-1726CAG-2017
	PLREKRRKRGLF	A-Duck-Egypt-1814-2018
	PLREKRRKRGLF	A-Common-coot-Egypt-CA285-2016
	PLREKRRKRGLF	A-green-winged_teal-Egypt-871-2016
	PLREKRRKRGLF	A-duck-Egypt-SS19-2017
	PLREKRRKRGLF	A-duck-Egypt-F446-2017
2.3.4.4A	PLRERRRKRGLF	A-chicken-Netherlands-emc-3-2014
	PLRERRRKRGLF	A-broiler-duck-Korea-H651-2014
	PLRERRRKRGLF	A-broiler-duck-Korea-Buan2-2014
	PLRERRRKRGLF	A-goose-Taiwan-TNO15-2015
	PLRERRRKRGLF	A-eurasian-wigeon-Netherlands-2-2014
	PLRERRRKRGLF	A-Chicken-Egypt-189CA-2018
	PLRERRRKRGLF	A-Chicken-Egypt1810CA-2018
	PLRERRRKRGLF	A-Chicken-Egypt-1812CA-2018

The table shows the difference between the cleavage site in group A and B with mutation K325R in red color. The mutation in K325R was found in group A and three samples isolated in 2018. Samples in bold are the samples of our study.

of 99 – 100% with A/chicken/Czech_republic/206-17_2/2017, 94.7 – 99.7% with A/tuffled/duck/Germany/AR8444 and AR8459/2016 represent to clade 2.3.4.4 group B Russian like reassortant. 96.7 – 98% A/goose/Shandong/WFSG1/2014 represent to clade 2.3.4.4 group B, 95.3%-96% with A-broiler-duck-Korea-Buan2-2014 and 94.3%-99% with A-chicken-Netherlands-emc-3-2014 represent to clade 2.3.4.4 group A (Figure 4).

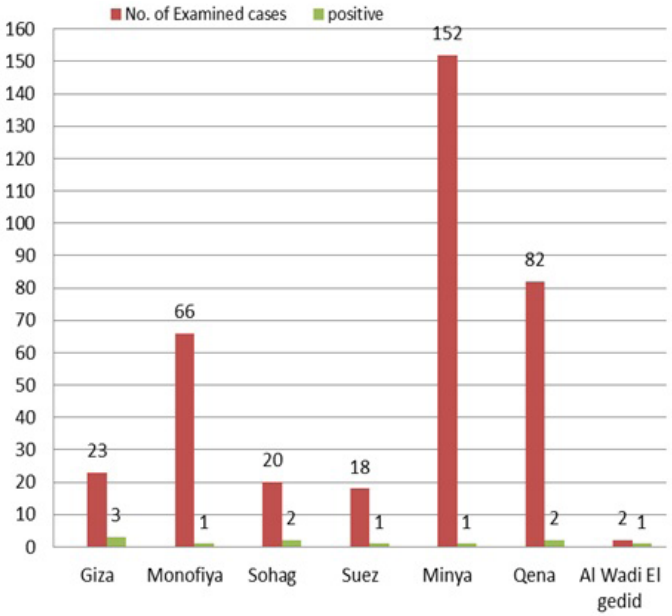

Figure 2: *Epidemic chart of HPAI H5N8 during May 2017-August 2018 in Egypt.* **FN:** *The Fig. show the number of examined and positive cases of HPAI H5N8 in different governorates.*

The highly pathogenic avian influenza virus (H5N8) in Egypt was firstly confirmed in wild bird from Domiatte governorate in north Egypt and it spreads rapidly in all sectors in Egypt causing high mortalities in poultry [31]. The virus was related to the Russia-Mongolia HPAI H5N8 viruses of clade 2.3.4.4b. [29]. Afterward, three different reassortant HPAI H5N8 viruses were detected in Egypt among wild and domestic birds indicating multiple introductions in 2017 [37]. The HPAI (H5N1) was entered and spread inside Egypt due to improper poultry infrastructure across poultry production sectors and marketing chains resulting in the endemicity of the disease [3], the same has been done with the highly pathogenic avian influenza H5N8 that entered and spread in Egypt from migratory bird to household causing high mortality in chicken industry [5].

In this study, the HPAI H5N8 virus was detected in eleven cases in seven governments with a prevalence rate of 0.09% from May 2017 to August 2018. These backyard spots are a potential source of HPAI H5N8 transmission to commercial farms and live bird markets and lead to higher economic losses due to the direct

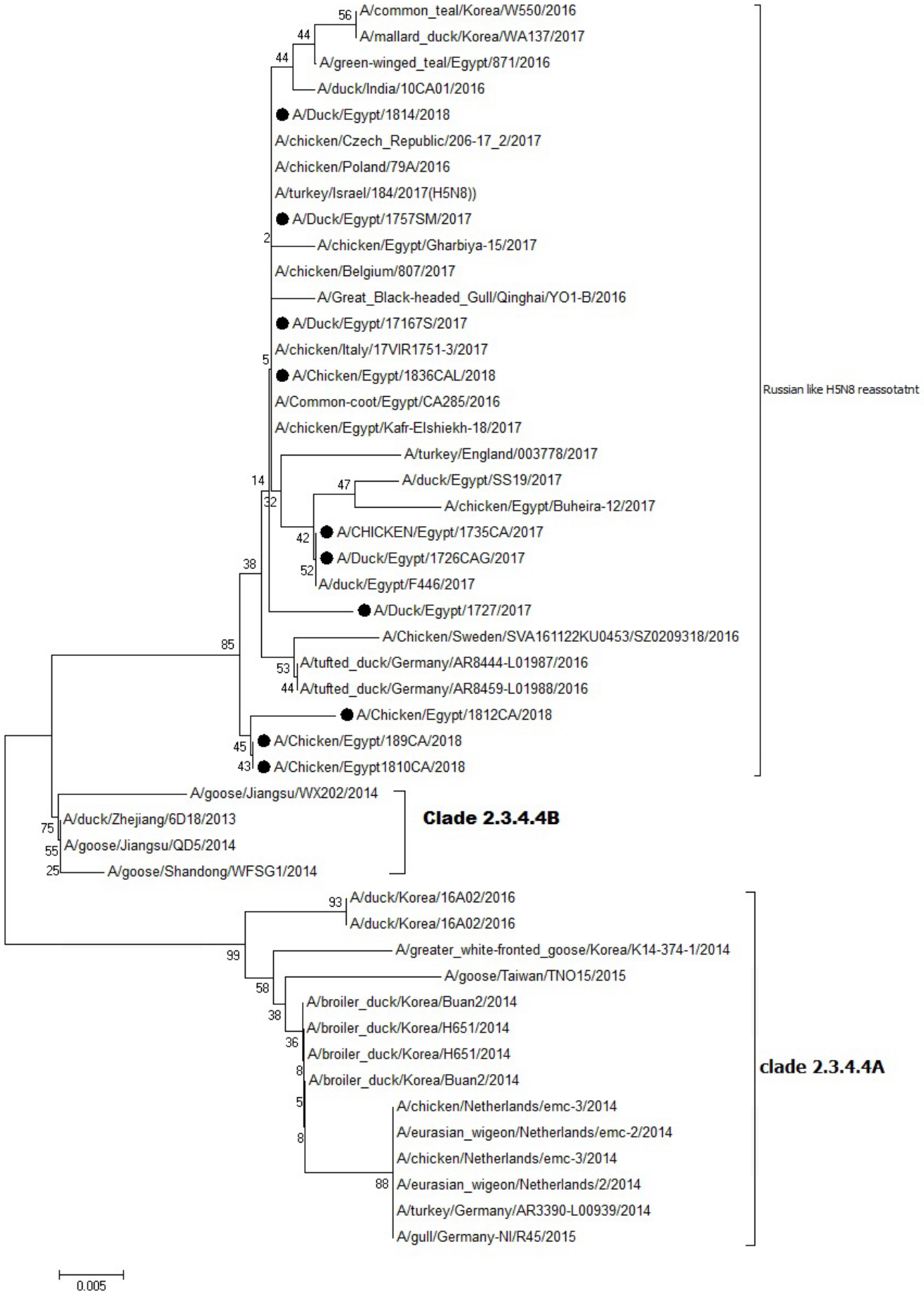

Figure 3: *Phylogenetic tree of the partial HA gene of HPAI H5N8* **FN:** *Fig. show all HA gene of positive samples included in AI H5N8 clade 2.3.4.4 group B. The sequenced viruses were clustered together in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4. black dots refer to the isolates of this study.*

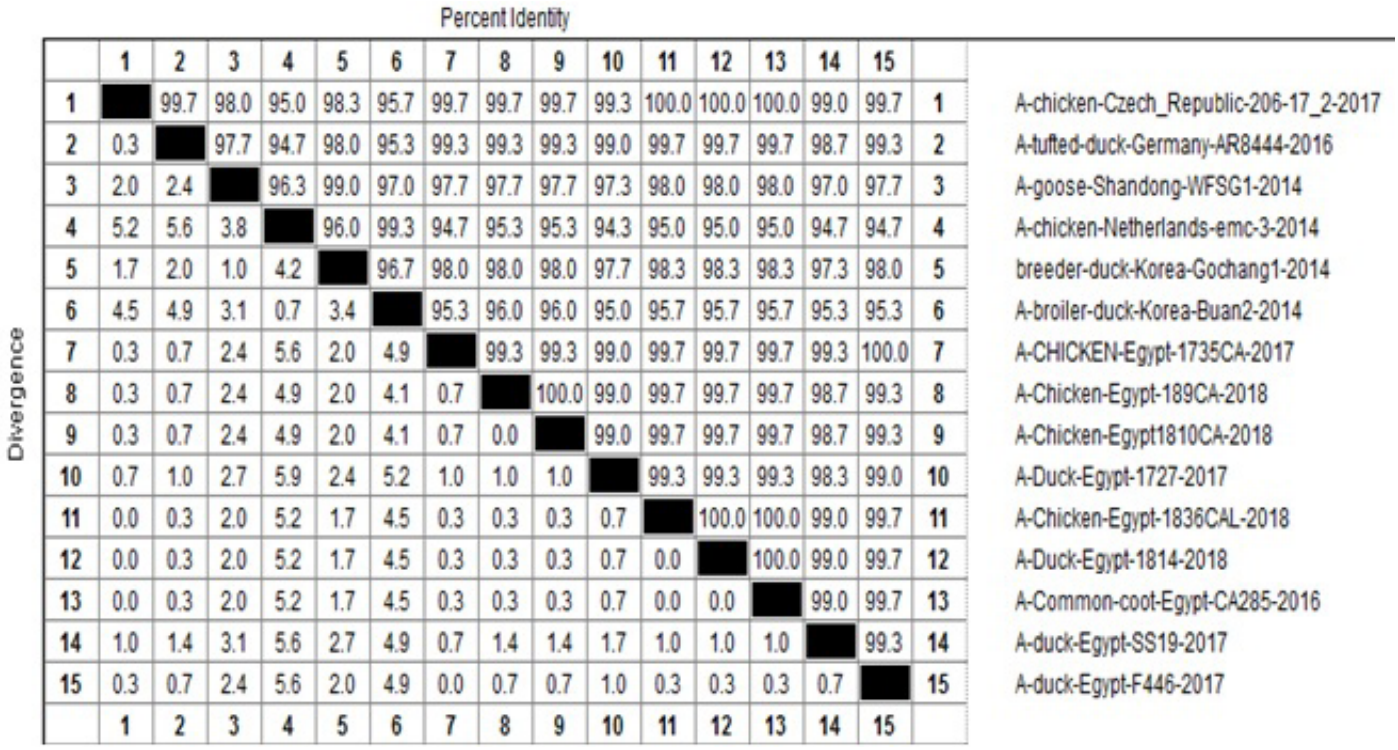

Figure 4: *Nucleotide identities and divergence of Sequenced viruses compared to other selected strains.* **FN:** *Comparative alignment of partial HA gene showed that HA genes of the 10 viruses with reference strains in the genebank.*

contact between backyard birds and small commercial poultry farms as previously recorded in the spread of avian influenza H5N1[11]. The marketing system [random uncontrolled movement of birds to/from live bird markets], and farm worker in commercial farms usually raise backyard birds in their houses [28].

The epidemiological analysis of our data revealed that the distribution of the HPAI H5N8 positive cases was more spreading in upper Egypt and the highest record of the positive cases were in November and December [during winter] and in April during spring due to the virus availability increase in low temperature in winter. The HPAI H5N1 was detected in Upper Egypt with high incidence in winter than any other time of year during 2015-2016 [14]. This result coincides with WHO/OIE/FAO H5N1 Evolution Working Group who declared that H5N1 AI became endemic in Egypt resulting in winter season lead to severe losses in the poultry industry [36].

In this study we sequenced 10 samples out of 11 positive cases of backyard represent seven governments in Egypt. The Phylogenetic analysis of the partial HA gene of the Egyptian H5N8 viruses indicated that they clustered together in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4. (Figure 3) and this was similar to the results of Yehia et al., 2017 [37] which showed the three strains of HPAI H5N8 represent to backyard and farms in Egypt in 2017 cluster to group B Russian like reassortant H5N8 viruses of clade 2.3.4.4b. In addition, the phylogenetic analysis of our sequenced viruses was agreed to that of Selim et al., 2017 [29] which indicated that the HA and NA gene sequences of the Egyptian virus revealed that it is clustered with clade 2.3.4.4b, along with the recent viruses widely distributed throughout Europe. Also, these results agreed with kandeil et al., 2017 [18] who provided detailed genetic characterization of Egyptian H5N8 viruses and demonstrated that the Egyptian H5N8 viruses are highly pathogenic avian influenza viruses and that the genome of the Egyptian H5N8 viruses was related to recently characterized reassortant H5N8 viruses of clade 2.3.4.4 isolated from different Eurasian countries.

The amino acid sequence of the protease cleavage site of HA protein from migratory bird revealed multiple basic amino acids, which is characteristic of HPAIV [18]. The multibasic cleavage sites of the HA of HPAI (H5N8) of seven strains were PLREKRRKR/GLF similar to group B of H5N8 viruses of clade 2.3.4.4b. (Table 2) as previously described [29, 18]. The three strains of HPAI (H5N8) in 2018 were PLRERRRKR/GLF similar to group A of H5N8 viruses of clade 2.3.4.4a as mentioned before [19].

The difference between the cleavage site of Gochang1 represents to HPAI (H5N8) clade 2.3.4.4. group B was LREKRRKR/GLF and Buan2 represent to HPAI (H5N8) clade 2.3.4.4. group A was, LRERRRKR/GLF, the two viruses were highly pathogenic to chicken with an intravenous pathogenicity index 3 [19, 33].

In conclusion, the surveillance of H5N8 highly pathogenic avian influenza during the period from May 2017 to August 2018 in backyard poultry production sectors revealed that the virus was recorded in higher percentage in Upper Egypt. The sequenced viruses were clustered together in group B Russian like reassortant H5N8 viruses of clade 2.3.4.4. The HA cleavage site was the same as clade 2.3.4.4b except three isolates in 2018 has different cleavage site similar to clade 2.3.4.4A.

We recommended continuous surveillance of backyard and nearby commercial farms and markets to follow up on the situation of HPAI H5N8 in Egypt. Furthermore, it is recommended to conduct a trial to study the effect of the mutation in the cleavage site in the pathogenicity and immunogenicity.

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Authors Contribution

Wesam Mady, Nahed Yehia, Mohammed Hassan Elhussieny, Azhar Gaber, Moataz Mohamed El Sayed, Neveen Rabie Bakry, Asmaa Shaaban, and Afaf Abdel Baset Mahmoud performed the research; Wesam Mady, Nahed Yehia, Mohammed Hassan Elhussieny, Azhar Gaber, Moataz Mohamed El Sayed, Neveen Rabie Bakry, Asmaa Shaaban, Abdel Satar Arafa, Afaf Abdel Baset Mahmoud, Wafaa Mohammed Mohammed Hassan were involved in drafting the work and revising it critically for important intellectual content. Wesam Mady, Nahed Yehia and Mohammed Hassan Elhussieny wrote the manuscript. And Wesam Mady, Nahed Yehia, Mohammed Hassan Elhussieny, Azhar Gaber, Moataz Mohamed El Sayed, Neveen Rabie Bakry, Asmaa Shaaban, Abdel Satar Arafa, Afaf Abdel Baset Mahmoud, Wafaa Mohammed Mohammed Hassan compiled the final approved version to be published.

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