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Thesis Review

# Avian influenza infections in poultry farms in Egypt, a continuous challenge: Current problems related to pathogenesis, epidemiology, and diagnosis

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GMPC-TOP
**Article History:**
**Received:** 27-Dec-20
**Accepted:** 29-Jan-21
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**Abstract**

This study's main objective was to update avian influenza (AI) epidemiological situation, including molecular characterization reassortment analysis and genotyping of circulating AI virus (AIV) subtypes in Egyptian poultry farms between 2017 and 2019. As a necessity for such work, improved diagnostic tools were developed for AIV detection. Subtype H9N2 infections were detected in 27 out of 39 examined farms and were frequently mixed with high pathogenic avian influenza (HPAI) H5N8 in 22/39 farms. Next-generation and Sanger sequencing helped to define novel reassortant HPAI H5N2 and low pathogenic avian influenza (LPAI) H9N2 in Egypt. Systematic reassortment analysis confirmed at least seven genotypes of HPAI H5Nx viruses of clade 2.3.4.4b and three genotypes of LPAI H9N2 circulating in Egypt. Several internal genes of AIVs previously detected in wild birds in Egypt were represented in the genome of novel reassortants of both HP H5Nx and H9N2 viruses suggesting local reassortment processes.

**Keywords:** Avian influenza, Egypt, reassortment, HPAI-H5N2, HPAI-H5N8

**Citation:** Hassan, K.E., Harder, T., and Hafez H. M. Avian influenza infections in poultry farms in Egypt, a continuous challenge: Current problems related to pathogenesis, epidemiology, and diagnosis. GMPC-TOP. 2021. 1 (1):12-16. https://doi.org/10.51585/gtop.2021.0004

## Introduction

Avian influenza (AI) in Egypt represents a huge challenge for poultry production due to different virus subtypes simultaneously at the same poultry farms. Egypt has a long history of AI: High pathogenic avian influenza (HPAI) viruses of subtype H5N1(clade 2.2) were firstly detected in 2006, while in 2010, low pathogenic avian influenza (LPAI) H9N2 was detected in quails, and since that date, both viruses were circulating in poultry farms **(El-Zoghby et al., 2012)**. The widespread of zoonotic HPAI H5N1 in Egypt had also threatened human health, causing 41.8% of all reported human cases with HPAI H5N1 worldwide. In early 2017, a new HPAI H5N8 virus of clade 2.3.4.4b was isolated from duck samples taken at the backyard and commercial farms in Egypt and suggesting multiple virus introductions through migratory wild birds **(Yehia et al., 2018)**.

Interestingly, gene segments LPAI viruses like the H7N3 virus, previously detected in wild birds in Egypt, were found in HPAI H5N8 viruses circulating in domestic poultry farms in Egypt **(Naguib et al., 2019)**. Recently, a new reassortant HPAI H5N2 was detected in a duck farm in Egypt **(Hagag et al., 2019)**. These findings illustrate that control measures to stop AI spread and circulation in poultry farms in Egypt were unsuccessful.

Along these lines, the presented study was conducted to update the AI epidemiological situation, including molecular characterization, reassortment analysis and genotyping of circulating AIV subtypes in Egyptian poultry farms in the period between 2017 and 2019.

## Materials and Methods

### *Samples collection and preparation*

During this study, samples were collected from commercial poultry farms suffering from respiratory disease manifestations between 2017 and 2019. All the sampled poultry flocks showed severe respiratory signs

with moderate mortality. Swabs were collected from clinically diseased birds of different species, including chicken (broiler, commercial layer, breeder and native broiler farms), duck, and turkey flocks from six Egyptian governorates, including Giza, Qualiobia, Fayoum, El-Menia, Dakahlia, and Beni-Suef. Swabs from each flock were pooled and resuspended in 2 ml of phosphate buffer saline (PBS) pH 7.0-7.4, clarified by centrifugation at 4000 rpm for 10 min and processed for RNA extraction **(OIE, 2014 )**. All samples were examined for AIV, velogenic New Castle disease virus (vNDV) and infectious bronchitis virus (IBV) infections by real-time RT-PCR (RT-qPCR).

***RNA extraction, virus detection and subtyping***

RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were initially tested for the presence of AIV RNA by one-step RT-qPCR targeting the M gene of influenza type A viruses **(Fereidouni et al., 2012)** using the AgPath real-time kit (Ambion) on a CFX96 thermocycler machine (Bio-Rad). Positive samples were further subtyped for hemagglutinin (HA) and neuraminidase (NA) using the so-called Riems influenza a typing array (RITA) RT-qPCR as described by **(Hoffmann et al., 2016)**. All samples were also examined for IBV by a generic RT-qPCR as previously described **(Naguib et al. 2015)** and NDV by using F gene-specific primers to detect the vNDV genotype VII-d **(Moharam et al., 2019)**.

***Sequencing of viral genome segments***

Both Sanger and next-generation sequencing were used in this study. Full HA and NA genes of H5 and H9 AI viruses were Sanger-sequenced using specific primers described previously **(Naguib et al., 2015)** using conventional one-step RT-PCR. Products were size-separated by agarose electrophoresis, fragments of correct lengths were excised and purified from gel using the QIAquick gel extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were subjected directly to cycle sequencing reactions (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems. The MinION method of next-generation sequencing was used for full genome sequencing of selected AIV isolates **(King et al., 2020)**.

***Sequence and phylogenetic analysis***

Sequences were assembled and edited using the Geneious software, version 11.1.7 **(Kearse et al., 2012)**. Alignment and identity matrix analyses were performed with Multiple Alignment using Fast Fourier Transform (MAFFT). Phylogenetic analyses were based on manually edited alignments of the full-length open reading frames. For maximum likelihood (ML) analysis of the phylogenetic relationship, the best fit model was chosen according to the Bayesian selection criterion calculated using ModelFinder included in the IQ-tree software version 1.6.9 **(Minh et al., 2013, Nguyen et al., 2015)**. To assess the ML trees' branch support, both an ultrafast bootstrap approximation UFBoot2 **(Hoang et al., 2018)** and the SH-aLRT test were run, each with 1000 replicates. UFBoot and SH-aLRT values of >95 and >80, respectively, were interpreted as a reliable indication of clade separation. Finally, trees were viewed and edited using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.92.

Full genome sequence data for 23 complete genomes of HPAI H5N8 sampled from Egypt during the period 2016–2020 (including the 3 new virus isolates of this study) in addition to two reassortant HP H5N2 virus genomes (A/duck/Egypt/VG1099/ 2018, A/chicken/Egypt/ Al00994/2019, further referred to as H5N2-A and -B) were downloaded from the EpiFlu database. Sequence alignments were trimmed to represent the major coding region of each gene segment (PB2, 2,280 nt; PB1, 2,274 nt; PA, 2,152 nt; HA, 1,703 nt; NP, 1,496 nt; NA, 1,413 nt; MP, 981 nt; NS, 838 nt) as well as an alignment of the concatenated major coding regions constructed for all 24 isolates (13,136 nt) were generated. Similarly, sequence data were arranged for 52 complete genomes of influenza A virus (H9N2) sampled from Egypt during 2010–2020. Major coding regions of each H9N2 segment including PB2 (2,227 nt), PB1 (2,253 nt; PA, 2,151 nt; HA, 1,682 nt; NP, 1,496 nt; NA, 1,411 nt); MP (978 nt); NS (814 nt); and concatenated major coding regions (13,012 nt) were constructed.

**Results and Discussion**

Various combinations of respiratory virus co-infections were detected that reflect the Egyptian poultry production sector's ongoing problems. The previously dominating HPAI H5N1 (clade 2.2.1) viruses were sporadically detected; meanwhile, the most detected combination was co-infection between HPAI-H5N8 and LPAI-H9N2 **(Figure 1)**.

Phylogenetic analyses of the HA protein showed that both H5 and H9 sequences were located at the tips of the respective cluster branches revealing ongoing genetic drift. Improved molecular diagnostic tools (RT-qPCR) had to be designed for recent H9N2 viruses due to several mismatches at the binding sites of previously used primers and probes. Full genome sequencing of AIV detected in the surveillance studies showed the circulation of new reassortants. Several new reassortant HPAI H5N8 viruses were identified, and novel reassortant LPAI H9N2 viruses in chicken farms.

Moreover, a novel reassortant HPAI H5N2 was detected for the first time in chicken farms in Egypt (**Figure 2).** The novel reassortant HPAI H5N2 virus-encoded the HA gene segment of HPAI H5 clade 2.3.4.4b viruses, with four genome segments (PB1, PB2, PA, and NS) obtained from another novel reassortant H9N2 virus firstly detected in pigeons in 2014 while the NP, NA, and M segments derived from classical H9N2 viruses circulating poultry farms in Egypt since 2010.

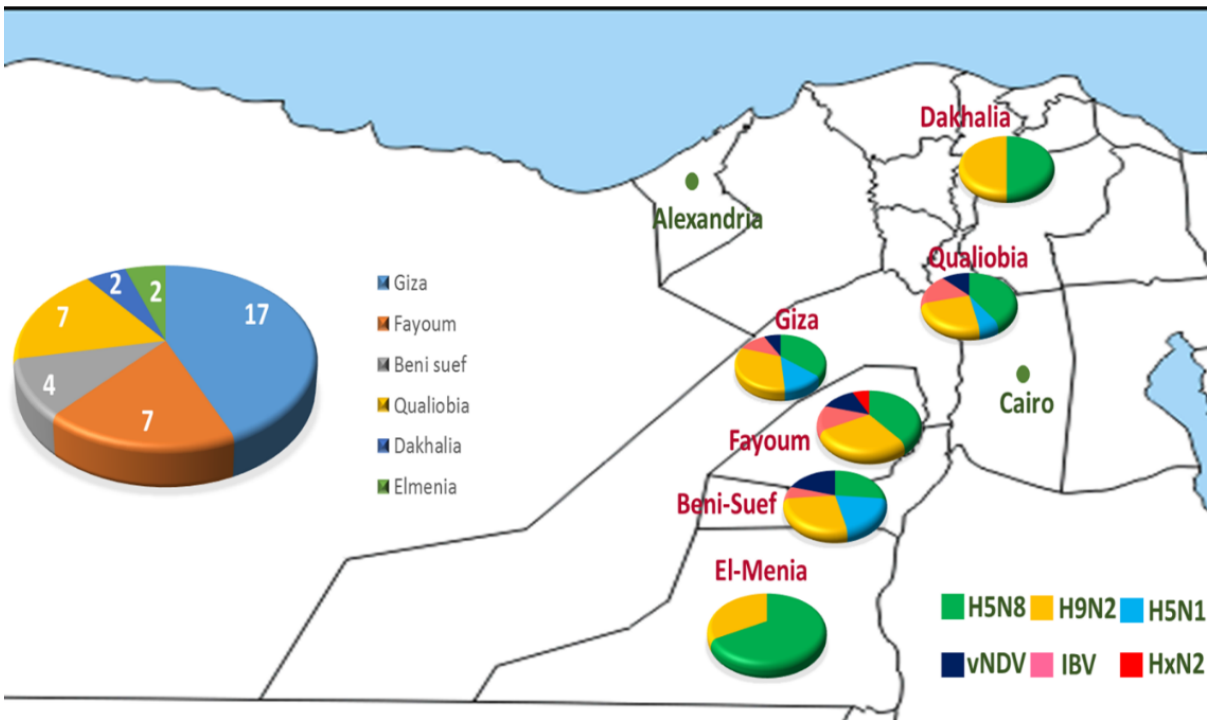


**Figure 1.** Geographical distribution of detected viruses during the study conducted during 2017-2018

Thus, this H5N2 reassortant virus is different from the one described in ducks in Egypt at about the same time. Comparing all available full genome sequences of Egyptian HPAI H5N8, and H5N2 and LPAI H9N2 viruses revealed the presence of seven HPAI H5Nx genotypes of clade 2.3.4.4b successively circulating in Egypt since 2016 **(Figure 2)**. Also, three different genotypes of LPAI H9N2 were discerned. Several internal genome segments of these genotypes related to LPAIs previously detected in wild birds in Egypt at the same time or earlier, including LPAI H7N9, H7N3, and H3N6 **(Kayed et al., 2019)**.

As another result of full-genome sequencing, amino acid mutations were detected for the first time in three HPAI H5N8 viruses in Egypt in the M2 and NA proteins proposing resistance to antiviral drugs Amantadine and Oseltamivir.

The most reliable way to confine the human population's exposure to zoonotic animal viruses is to prevent the spread of such viruses from their reservoirs to and within livestock animal populations **(Wang and Crameri, 2014).** In Egypt, enzootic cycles of AIV in poultry continue to pose a significant potential threat to public health. Appreciable success in combatting enzootic AIV needs a concerted national control strategy based on the cooperation of different sectors, including the Ministries of Agriculture (Veterinary Authorities), Health, Interior Affairs, accompanied by Presidential authority.

## Conclusions

Ongoing co-circulation of avian respiratory viruses in Egypt, including different AIV subtypes, vND, and IBV viruses, has exacerbated clinical signs (respiratory complex syndrome) in poultry flocks. This convoluted intervention and control measures. The HPAI H5N8 of clade 2.3.4.4b dominated the epidemiologic situation and has mostly replaced the potentially zoonotic HPAI H5N1. This may be a key factor explaining the lack of reported HPAI H5 human infections since 2018 in Egypt.

Expansion of viral variability exemplified by detecting novel reassortant HPAI H5N2 and LPAI H9N2 viruses in chickens re-emphasized the urgent need for a long-term solution combatting AIV infections in poultry. This ultimately aims at preventing further emergence of potentially zoonotic avian influenza viruses. Continuous checking and updating of molecular diagnostic tools is of utmost importance to avoid escaping the detection of emerging mutant viruses.

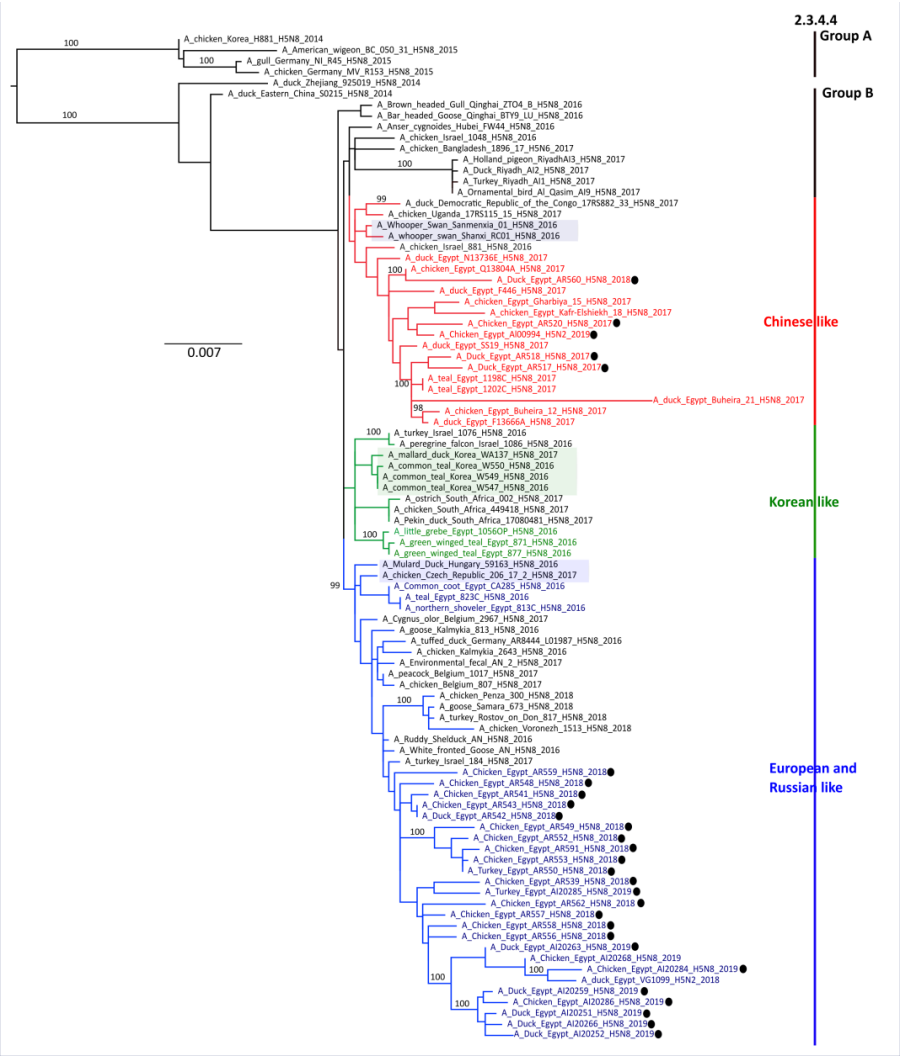


**Figure 2.** Phylogenetic analysis of the hemagglutinin segments of reassortant highly pathogenic avian influenza H5N2 and H5N8 viruses belonging to clade 2.3.3.4b from Egypt and reference viruses. Sequence analysis was based on alignment analyses by MAFFT version 7.450 in the Geneious software suite, version 11.1.7 (https://www.geneious.com) with manual editing. We performed maximum-likelihood calculations using PhyML version 3.0 (http:// www.atgc-montpellier.fr/phyml); we chose the best-fit model according to the Bayesian selection criterion using Model Finder embedded in Geneious. Entries with black dots depict sequences established in this study. Groups were colored according to their geographic origin: Red – Chinese; Blue - European and Russian; Green – Korean. The same color of the branches highlights Egyptian origin viruses in the respective groups. Clade designation is shown to the right of the HA phylogenetic tree. GenBank or GISAID accession numbers (http://www.gisaid.org) are provided for reference sequences. Scale bar indicates nucleotide substitutions per site

**Additional Article Information**

**Funding.** Kareem E. Hassan received a grant from the Egyptian Ministry of Higher Education and Scientific Research (Mission sector)

**Acknowledgment.** We acknowledge Prof. Magdy Fathy EL-Kady, professor of poultry diseases at Beni-Suef University, for providing samples and permissions to transfer samples from Egypt to Germany. Also, we acknowledge the originating and submitting laboratories that provided sequences available in the EpiFlu database (http://www. gisaid.org).

**Conflict of Interest.** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this work.

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- **Awarding University:** Poultry Diseases Institute, Free University of Berlin, Germany
- **Graduation year:** 2020
- **Language:** English
- **Thesis title:** Avian influenza infections in poultry farms in Egypt, a continuous challenge: Current problems related to pathogenesis, epidemiology, and diagnosis.
- **Link to the full thesis:** https://refubium.fu-berlin.de/handle/fub188/27881

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