Molecular identification of avian influenza virus subtypes H5N1 and H9N2 in birds from farms and live bird markets and in respiratory patients

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Background. Avian influenza viruses (AIVs) have been endemic in Egypt since 2006, and the co-circulation of high-pathogenic avian influenza H5N1 and low-pathogenic avian influenza H9N2 subtypes in poultry has been reported; therefore, Egypt is considered a hotspot for the generation of new subtypes and genotypes. We aimed to characterize AIVs circulating on commercial farms and in live bird markets (LBMs) during the winters of 2015 and 2016 in the study area and to identify H5N1 and H9N2 viruses in respiratory patients. **Methods.** In total, 159 samples were collected from ducks, pigeons and quails on farms (n = 59) and in LBMs (n = 100) and screened by real-time RT-PCR for H5N1 and H9N2 subtypes. Clinical and postmortem examination was carried out on birds from the farms. Positive H5N1 samples were sequenced and analysed for mutations. Tracheal swabs were also collected from 89 respiratory patients admitted to respiratory hospitals in the same study area. **Results.** Overall, H5N1 was identified in 13.6% of birds from farms, while it was detected in 17% of birds in LBMs. Subtype H9N2 was only identified from pigeons on farms (6.5%) and LBMs (11.4%). Sequencing of the haemagglutination gene (HA) in nine representative H5N1 isolates revealed a multi-basic amino acid motif at the cleavage site (321-PQGEKRRKKR/GLF-333), which is characteristic of highly pathogenic AIV, in five of our isolates, while the other four isolates showed an amino acid substitution (Q322K) at this cleavage site to make it (321-P K GEKRRKKR/GLF-333). All the isolates belonged to clade 2.2.1.2, and a comparison of HA sequences at the amino acid level showed 98.8-100% homology among the nine isolates, while they showed 94.1-96.1% identity with reference strains and the commonly used vaccine strain in Egypt. Out of 89 respiratory patients, 3.4% were positive for H5N1 and no patients were positive for H9N2. **Discussion.** Our results indicated the circulation of the endemic H5N1 and H9N2 viruses among poultry in 2015 and 2016. Birds on farms and in LBMs are reservoirs playing a role in the

dissemination of the virus and producing a public health risk. The application of proper hygienic measures in farms and LBMs to control the exposure of birds and humans to the source of infection along with continuous monitoring of the circulating viruses will provide information on understanding the evolution of the viruses for vaccine studies.

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Abstract

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Methods. In total, 159 samples were collected from ducks, pigeons and quails on farms (n = 59) and in LBMs (n = 100) and screened by real-time RT-PCR for H5N1 and H9N2 subtypes. Clinical and postmortem examination was carried out on birds from the farms. Positive H5N1 samples were sequenced and analysed for mutations. Tracheal swabs were also collected from 89 respiratory patients admitted to respiratory hospitals in the same study area.

Results. Overall, H5N1 was identified in 13.6% of birds from farms, while it was detected in 17% of birds in LBMs. Subtype H9N2 was only identified from pigeons on farms (6.5%) and LBMs (11.4%). Sequencing of the haemagglutination gene (HA) in nine representative H5N1 isolates revealed a multi-basic amino acid motif at the cleavage site (321-PQGEKRRKKR/GLF-333), which is characteristic of highly pathogenic AIV, in five of our isolates, while the other four isolates showed an amino acid substitution (Q322K) at this cleavage site to make it (321-PKGEKRRKKR/GLF-333). All the isolates belonged to clade 2.2.1.2, and a comparison of HA sequences at the amino acid level showed 98.8-100% homology among the nine isolates, while they showed 94.1-96.1% identity with reference strains and the commonly used vaccine strain in Egypt. Out of 89 respiratory patients, 3.4% were positive for H5N1 and no patients were positive for H9N2.

Discussion. Our results indicated the circulation of the endemic H5N1 and H9N2 viruses among poultry in 2015 and 2016. Birds on farms and in LBMs are reservoirs playing a role in the dissemination of the virus and producing a public health risk. The application of proper hygienic measures in farms and LBMs to control the exposure of birds and humans to the source of infection along with continuous monitoring of the circulating viruses will provide information on understanding the evolution of the viruses for vaccine studies.

Introduction

Influenza A viruses belong to the family *Orthomyxoviridae* and are categorized into 16 haemagglutinin (HA) subtypes and nine neuraminidase (NA) types in wild and domestic birds (Fouchier et al. 2005). To date, genetic material of influenza viruses of the H17N10 and H18N11 subtypes has been detected in bats (Tong et al. 2012; Tong et al. 2013). In poultry, avian influenza viruses (AIVs) are classified according to the severity of the caused disease as highly pathogenic (HP) and low pathogenic (LP) viruses (Swayne & Suarez 2000). Infection with LPAIVs is usually benign, including mild respiratory signs with/without a reduction in egg production. However, HPAIVs (especially H5 and H7 subtypes) may cause 100% mortality (Alexander 2007).

The poultry industry in Egypt consists of two major sectors: commercial (estimated 850 million birds) and backyard (estimated 250 million birds) (Abdelwhab et al. 2010). As a result of insufficient poultry slaughterhouses and the preference for consuming freshly slaughtered birds, the poultry meat trade depends mainly on live bird markets (LBMs) selling live or slaughtered birds for consumers (Abdelwhab et al. 2010). There are two types of LBMs: small retail outlets that depend mainly on commercial poultry and, to a lesser extent, those that sell home-reared birds. The second type of LBM is a large market in each city and village that is held once per week and

includes different vendors for selling birds, vegetables, seeds and other food. Birds of different species (chicken, ducks, geese, quail, pigeons) are usually put together in the same cage (Abdelwhab et al. 2010). The biosecurity standards and veterinary inspections in LBMs are minimal or completely absent. Birds in LBMs originate from different sources, such as farms and home rearing at various localities; which resulted in amplification and dissemination of the virus and increasing the probability of transmission to other birds and humans (Cardona et al. 2009). LBMs are considered critical links for commercial farms, slaughter houses, traders and final consumers (Ibrahim et al. 2007).

Since the H5N1 outbreak in Egypt in mid-February 2006, enormous loss in the poultry industry has occurred, and the slaughter campaign overwhelmed the resources of veterinary and public health authorities (Abdelwhab & Hafez 2011). Despite vaccination and biosecurity measures have been implemented, the disease is still endemic in Egypt and affecting the poultry and public health sectors. Multiple clades of Egyptian A/H5N1 have been reported with at least two distinct genotypes: the 2.2.1.1 clade isolated mainly from vaccinated commercial poultry (and rarely from backyard birds) and the 2.2.1/C virus isolated from backyard birds, small-scale commercial poultry and humans (Abdelwhab et al. 2012; Arafa et al. 2012a). Clade 2.2.1.2 has predominated in Egypt since 2011, and the virus has mutations associated with increased binding affinity to human receptors, thus posing a public health risk (Arafa et al. 2016a).

In addition to the economically disastrous effect of AIV on the poultry industry, public health concerns arose due to the zoonotic potential of HPAI H5N1 (Naguib et al. 2015). Worldwide, during the 2006 outbreak of HPAI H5N1, 202 persons were infected, and 113 died; most of these victims were in direct contact with infected or dead birds (Wang et al. 2006; WHO 2006). In Egypt, since 2003 till 2017, 359 cases and 120 deaths were reported due to H5N1 (WHO 2017). Most

92 studies relied on seroprevalence to determine which human cases had the H5N1 subtype (Gomaa
93 et al. 2015). Poultry infected with the H9N2 AIV are usually asymptomatic or show mild clinical
94 signs; however, frequent outbreaks of the virus have been reported in Asia and the Middle East
95 with high mortality (Guo et al. 2000; Naeem et al. 1999), Morocco (EL Houadfi et al., 2016) and
96 Burkina Faso (Zecchin et al., 2017). The co-circulation of HPAI H5N1 and LPAI H9N2 has added
97 an additional risk factor to the poultry industry and humans (Arafa et al. 2012c). Reassortment of
98 these LPAI viruses with HPAI H5N1 could produce novel influenza viruses capable of crossing
99 the interspecies barrier and causing zoonotic transmission and infection in humans (Turner et al.
100 2017). The isolation of H9N2 virus from humans in contact with poultry has been reported
101 indicating its zoonotic potential (Kalthoff et al. 2010; Turner et al. 2017). In Egypt, since 2010,
102 H9N2 has become endemic; furthermore, in 2015, the first human H9N2 case was reported
103 (Naguib et al. 2015). Therefore, this study aimed to characterize AIVs circulating on commercial
104 farms and in LBMs during 2015-2016 in the study area and to identify the H5N1 and H9N2 viruses
105 in respiratory patients.
106
107 **Materials and Methods**
108 **Sampling and data collection**
109 Duck, pigeon and quail samples were collected during the winters of 2015 and 2016 from farms
110 and LBMs in Sharkia Governorate, Egypt. The birds in LBMs were housed in the same cages. The
111 birds from farms were those admitted to the Clinic of Avian and Rabbit Medicine Department, Faculty
112 of Veterinary Medicine, Zagazig University, Egypt. Birds admitted to the clinic were subjected to
113 clinical and postmortem examination. The number of sampled birds from LBMs and farms,
114 respectively, included ducks (45 and 24), pigeons (35 and 31) and quail (20 and 4). None of the birds

from farms or LBMs received AIV vaccines except one duck sample from a farm (collected in 2015). Tracheal swabs from birds in LBMs were pooled (each pool consisted of swabs from 5 birds) and collected in tubes containing 2-3 mL viral transporting medium (VTM), which consists of phosphate-buffered saline (PBS) (Biowhittaker) and PEN-STREP (10000 U penicillin/ml–10000 U streptomycin/ml, Biowhittaker, lonza). From each bird admitted to the clinic, the brain, liver and lungs were pooled and homogenized in 10 mL sterile PBS. The homogenate was then centrifuged at 2000 rpm for 20 min at 4°C. The supernatant was pipetted and transferred to sterile Eppendorf tubes containing 100 µL PEN-STREP antibiotic and stored at -20°C until used for real-time RT-PCR and virus isolation.

Throat swabs from 89 respiratory patients admitted to respiratory clinics in general hospitals in Sharkia Governorate, Egypt, were collected in VTM tubes. In addition to the sample collection, an epidemiological questionnaire was conducted to collect information about patients, including sociodemographic information (sex, age, occupation, residence, and education level), level of knowledge about AI, attitudes and practices. Informed verbal/written consent for participation in the study was obtained from all the participants, and the study was approved by the Committee of Animal Welfare and Research Ethics (protocol #12/2015), Faculty of Veterinary Medicine, Zagazig University, Egypt.

Virus isolation

Swabs and tissue homogenates from the examined birds were injected into 9-11-day-old specific pathogen-free embryonated chicken eggs via the allantoic route according to recommendations of the OIE (2012). The allantoic fluid was collected from eggs with dead

137 embryos after 48 h and screened by slide haemagglutination test. At least three successive embryo
138 passages were applied for each sample to be negative.
139
140 **Real time RT-PCR** Viral RNA from HA allantoic fluids (bird samples) and from human
141 swabs was extracted using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) following
142 the manufacturer's guidelines. Primers and probes supplied from Metabion (Germany) targeting
143 the H5N1 (Slomka et al. 2009) and H9N2 (Ben Shabat et al. 2010) subtypes were used
144 (Supplementary Table S1). Real-time RT-PCR amplifications were performed in a final volume
145 of 25 µL containing 7 µL of RNA template, 12.5 µL of 2x QuantiTect Probe RT-PCR Master Mix
146 (Qiagen, Germany), 3.625 µL PCR-grade water, 0.25 µL of each primer (50 pmol), 0.125 µL of
147 each probe (30 pmol) and 0.25 µL of QuantiTect RT Mix. Reverse transcription was performed at
148 50°C for 30 min, followed by primary denaturation at 94°C for 15 min, followed by 40 cycles of
149 denaturation at 94°C for 15 s, annealing at 54°C for 30 s and extension at 72°C for 10 s. The
150 reaction was performed in a STRATAGENE MX3005P real-time RT-PCR machine. Samples that
151 were positive by H9N2 subtype real-time RT-PCR were further confirmed using the same primers
152 in a conventional RT-PCR reaction to assure that the resulting amplification had the correct
153 product size. Extracted RNA was transcribed to cDNA using the RevertAid H Minus First Strand
154 cDNA synthesis kit (Fermentas Inc., Maryland, USA) following the manufacturer's instructions.
155
156 **Sequence and phylogenetic analysis**
157 PCR amplification of the H5 gene segment spanning the cleavage site was carried out using
158 the primers and reaction conditions previously described (Njouom et al. 2008). Nine H5N1 isolates
159 from the positive bird samples were selected for sequencing. Amplicons of 311 bp were purified

160 using a QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) according to the
161 manufacturer's guidelines. Sequencing was then carried out using Bigdye Terminator V3.1 cycle
162 sequencing kits (Perkin-Elmer, Foster City, CA) in an Applied Biosystems 3130 genetic analyser
163 (HITACHI, Japan). The HA subtypes were identified by nucleotide BLAST
164 (http://www.ncbi.nlm.nih.gov/BLAST) and submitted to GenBank with accession numbers
165 KX228223, KX228224, KX228225 and KX228226 (winter 2015 samples) and MG725890,
166 MG725891, MG725892, MG725893 and MG725894 (winter 2016 samples). Alignment of the
167 nucleotide sequences with other HA gene sequences available in GenBank was performed by the
168 Clustal W method using the MegAlign module of DNAStar software (Lasergene version 7.2;
169 DNASTAR, Madison, WI, USA). The phylogenetic tree was generated using the neighbour-
170 joining method in MEGA version 7 (www.megasoftware.net). The tree topology was evaluated by
171 1000 bootstrap analyses.
172
173 **Statistical analysis**
174 Statistical analysis was performed using STATA version 15 for Windows (Stata Corp.,
175 USA). The prevalence of AIV infection in live birds and human patients was estimated from the
176 ratio of positive to the total number of samples tested. Because of the low number of cases in
177 respiratory patients, the unconditional associations between AIV infection and independent
178 variables (age, gender, occupation, residence, educational level, knowledge about AIV, route of
179 infection, transmission, preventive measures and vaccinations) were assessed by Fisher's exact
180 test. Further, correspondence analysis was used to analyse the relationship between the outcome
181 and categorized independent variables (Greenacre 2017).
182

183 **Results**

184 **Clinical and postmortem examination**

185 The farm birds examined in the study were obtained from cases admitted to the Clinic of
186 Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University. The
187 ducks showed general signs of illness, such as loss of appetite and decreased feed and water
188 consumption, and specific signs, such as nervous signs (twisting of the neck, trembling and
189 torticollis), respiratory signs (rhinitis, nasal and ocular discharge) and greenish watery diarrhoea.
190 Pigeons showed nervous signs, including twisting of the neck (**Fig. 1A**), inability to fly, tremors,
191 incoordination, circling in flight, flying backwards and torticollis. Respiratory signs in some cases
192 and greenish diarrhoea were also observed. None of the quails showed clinical signs except loss
193 of appetite and low egg production.

194 Postmortem examination of the ducks revealed congestion in the brain with petechial
195 haemorrhage, congestion in the liver and spleen with necrosis and haemorrhage in the pancreas
196 (**Figs. 1B, 1C and 1D**). In pigeons, the postmortem lesions showed septicaemia (congested
197 parenchymatous organs) in most organs with congestion in the brain (**Figs. 1E and 1F**). Quails
198 showed congestion in parenchymatous organs, with congestion in the brain. In female quails,
199 salpingitis and congestion in the uterus were observed.

200

201 **Virus isolation and haemagglutination**

202 Inoculation of embryonated chicken eggs with swabs and tissue homogenates from the bird
203 samples revealed mortality and AIV lesions in embryos within 24-48 h in all pigeons and 19 of
204 the duck samples of farm origin. Haemagglutinating viruses were detected in 13 (41.9%) pigeons
205 and 6 (25%) duck samples. With regard to samples from LBMs, inoculated eggs showed lesions

206 in 15 pigeons, 17 ducks and 4 quail pooled samples. Haemagglutination was observed in 13
207 (37.1%) pigeons, 14 (31.1%) ducks and one (5%) quail sample. The HA unit of infective AIV
208 allantoic fluids ranged from 1/64 to 1/1024
209
210 **Molecular identification**
211 Positive HA allantoic fluids from pigeon, duck and quail samples were subjected to reverse
212 transcription (RT) real-time RT-PCR for the identification of H5N1 and H9N2 subtypes. The
213 results revealed that the H5N1 subtype was identified in ducks (16.7%) and pigeons (12.9%) of
214 the farm origin, while H9N2 subtype was only identified in 6.5% of pigeons (**Table 1**). All the
215 samples were from non-vaccinated birds except one duck sample (collected in 2015).
216 Samples from LBMs identified the H5N1 subtype in 20% of both ducks and pigeons, while
217 only 5% of quails were positive by real-time RT-PCR (**Table 1**). Subtype H9N2 was identified in
218 11.4% of pigeons only. Overall, H5N1 subtype was identified in 13.6% of birds from farms, while
219 it was detected in 17% of birds from LBMs, which was a nonsignificant difference ($P = 0.73$). The
220 Ct values from all the examined samples are shown in Supplementary Table (S2).
221
222 **Sequence and phylogenetic analysis**
223 H5N1 isolates were further confirmed by sequencing and nucleotide BLAST analysis.
224 Sequencing of the HA segment revealed a multi-basic amino acid motif at the cleavage site (321-
225 PQGEKRRKKR/GLF-333), which is characteristic of HPAIV, in five of our isolates (**Fig. 2**). The
226 other four isolates showed an amino acid substitution (Q322K) at this cleavage site to make (321-
227 PKGEKRRKKR/GLF-333). In comparison with the reference (parent, classic and variant) strains
228 in Egypt, all the isolates in the current study showed the R325K amino acid substitution, while the

Q322K substitution was found in only four of our isolates compared to the reference and vaccine strains.

All the isolates belonged to clade 2.2.1.2 (**Fig. 3**), and a comparison of HA sequences at the amino acid level showed 98.8-100% homology among the nine isolates, while they showed 94.1-96.1% identity with reference strains and the commonly used vaccine strain (A/chicken/Egypt/Q1995D/2010) in Egypt. A lower similarity rate (90.2-91.6%) at the amino acid level was detected when compared with the A/duck/Potsdam/1402-6/1986 vaccine strain.

Duck isolates in the current study showed 98.8-100% amino acid homology with the latest circulating duck strains in Sharkia Governorate (A/duck/Egypt/1435CAS/2014) and in Egypt (A/duck/Egypt/2/2015). In contrast, pigeon and quail isolates showed 97.7-100% amino acid identity with the latest pigeon circulating strain in Sharkia Governorate (A/pigeon/Egypt/Sharkia-22/2014) and 100% with the only two quail circulating strains in Egypt (A/quail/Egypt/1171SG/2011) and (A/quail/Egypt/14102TCP/2014).

Human patients

A total of 89 respiratory patients (age range 10 – 74 years), with a higher proportion of females (62.9%) than males (37.1%), were involved in this study (**Table 2**). Approximately 84.3% of participants had heard about AIV, 56.2% were rural residents, and only 10.1% of participants applied AIV preventive measures. Examination of throat swabs from respiratory patients by real-time RT-PCR revealed that 3 out of 89 (3.4%) of the patients were positive for the H5N1 subtype, while none of the samples were positive for H9N2. **Fig. 4** illustrates the risk factors associated with AIV infection among the respiratory patients admitted to hospitals. Positive cases were closely associated with illiterate older persons (≥50 years) who live in rural areas. The graph also

252 highlights the close association between AIV cases and hygienic conditions (patients who did not
253 apply any AIV preventive measures). On the other hand, patients without AIV infection (negative
254 cases) were associated with young (≤19 years), educated, and hygienic persons who lived in urban
255 areas.
256
257 **Discussion**
258 The endemic status of H5N1 and H9N2 viruses in Egypt has resulted in outbreaks in poultry
259 and several human cases. The clinical examination of ducks naturally infected with H5N1 in our
260 study revealed nervous signs and diarrhoea, while gross lesions, including congestion and
261 haemorrhages, were observed in all internal organs. These were in accordance with different
262 studies in Egypt (Hafez et al. 2010; Hagag et al. 2015). In Korea, the same observation in naturally
263 infected ducks was reported (Rhyoo et al. 2015; Woo et al. 2011). The susceptibility of pigeons to
264 infection with HPAI H5N1 has been controversial since 2004; however, other studies have
265 documented that pigeons are experimentally susceptible to the virus and could serve as a source
266 of infection for other animals (Mansour et al. 2014; Smietanka et al. 2011; Songserm et al. 2006).
267 Clinical examination of pigeons in the present study revealed nervous signs, greenish diarrhoea
268 and congestion in internal organs. This was in agreement with a recent study that reported the first
269 natural infection of pigeons in Egypt (Mansour et al. 2014). The neurotropic nature of H5N1 in
270 pigeons has been proven experimentally, supporting the observed neurological signs (Klopfleisch
271 et al. 2006). Brain congestion was a predominant lesion in pigeons, and this finding has been
272 reported previously in other studies (Jia et al. 2008; Mansour et al. 2014).
273 In the current study, the isolation rate of H5N1 and H9N2 viruses from birds originating
274 from farms was insignificantly lower than the isolation rate from LBMs. Different studies have

compared the isolation rates of AIVs from farms and LBMs; for instance, the detection rate of H5N1 in commercial farm birds (0.1%) was significantly lower than that (11.4%) of LBMs (El-Zoghby et al. 2013). In contrast, a higher detection rate in commercial farms (6.8%) versus 3.3% in LBMs was also reported in Egypt (Kayali et al. 2011). The high positivity rate of AIVs in LBMs in our study (17%) was comparable to 11.5% (El-Zoghby et al. 2013) and 12.4% (Abdelwhab et al. 2010) in Egypt, while lower rates of 0.08% in Nigeria (Joannis et al. 2008), 1.3% in Thailand (Amonsin et al. 2008) and 3.3% in Egypt (Kayali et al. 2011) were also reported. In poultry from commercial farms, AIVs were identified in 13.6% of the samples; in contrast, lower positivity rates were reported in other studies in Egypt (El-Zoghby et al. 2013; Kayali et al. 2011).

The detection of HPAI H5N1 in birds from LBMs has an epidemiological impact due to several factors: mixing birds of different origins, species and ages reduces the ability to trace the source of infection; inter- and intra-species transmission of the virus occurs; and importantly, there is increased exposure of vendors, consumers and children to sources of infection (Abdelwhab et al. 2010). Housing and mixing birds in cages for sale in LBMs plays role in the propagation and dissemination of AIVs (Turner et al. 2017).

All positive samples from LBMs were from birds with no symptoms, thus indicating the vital role of asymptomatic birds in the persistence and dissemination of the virus (Abdelwhab et al. 2010; Amonsin et al. 2008; Nguyen et al. 2005). Consumers prefer to purchase waterfowl, especially ducks, from LBMs and backyard rearing rather than from commercial farms. The higher detection rate of H5N1 virus from ducks in LBMs indicated the importance of this bird as a silent asymptomatic carrier that plays a role in dissemination and maintenance of the virus for long periods (El-Zoghby et al. 2013; ElMasry et al. 2017; Hassan et al. 2013).

297 Pigeons have been reported to be susceptible to infection with HPAI H5N1 and LPAI H9N2
298 viruses and could be a source of infection for other animals and subsequently for humans due to
299 their abundance in backyard poultry farms (Mansour et al. 2014; Smietanka et al. 2011; Songserm
300 et al. 2006). In our study, H5N1 and H9N2 viruses were detected in pigeons from both farms and
301 LBMs. Studies reporting natural infection of pigeons are relatively scarce; however, experimental
302 studies have reported the susceptibility of pigeons to HPAIV and their ability to disseminate the
303 virus (Klopfleisch et al. 2006; Mansour et al. 2014; Smietanka et al. 2011). A few studies have
304 reported the detection of H5N1 virus from naturally infected pigeons in Egypt during 2009
305 (Elgendy et al. 2016) and 2014 (Mansour et al. 2014; Mansour et al. 2017). The present study
306 reported H5N1 infection in pigeons during 2015 and 2016 in Egypt. Subtype H9N2 was only
307 identified in pigeons from farms (6.5%) and LBMs (11.4%); these birds were also co-infected with
308 the H5N1 subtype. This was consistent with other studies in Egypt in different governorates
309 (Kayali et al. 2014; Njabo et al. 2016).

310 In the present study, none of the quails from farms were positive for AIVs, while only one
311 apparently healthy bird (5%) from a LBM was positive for H5N1. In accordance, a study
312 conducted in Egypt from 2005-2007 reported the isolation of H5N1 from quails (Mady et al. 2010).
313 Another study reported the confirmation of H5N1 from quails on farms (0.2%) and in LBMs
314 (0.8%) (Arafa et al. 2016b). Quails have been reported to be an important mixing vessel for AIVs
315 due to the expression of receptors for mammalian and avian AIVs on their epithelial cell surfaces
316 (Nguyen et al. 2016; Thontiravong et al. 2012). The infection of quails with HPAI H5N1 has also
317 been reported in different countries worldwide due to the intermingling of quails with other birds
318 in LBMs (Nguyen et al. 2016). None of the quails were positive for H9N2, and this finding was
319 also reported in a recent study (Zahida et al. 2017). In contrast to this finding, another study in

Egypt described the isolation of H9N2 from apparently healthy quail (Arafa et al. 2012b; El-Zoghby et al. 2012).

The co-existence of H5N1 and H9N2 subtypes in poultry has been reported, which has caused public health concerns because the endemic H9N2 viruses in Egypt contain elements that may favour avian-to-human transmission (Kandeil et al. 2017; Sun et al. 2011). Moreover, the co-circulation of the two subtypes has resulted in reassortment between the two subtypes, and thus, the reassorted virus has been associated with increased mortality and spread of infection among poultry (Kayali et al. 2014). Therefore, Egypt is considered a hotspot for the generation of new subtypes and genotypes (Abdelwhab & Abdel-Moneim 2015).

The isolates of the current study revealed the presence of a multi-basic amino acid sequence "EKRRKKR/GLF" at the proteolytic cleavage site, which is characteristic of HPAIV. This was consistent with other studies in the same area (Arafa et al. 2016a; ElBakrey et al. 2015; Mansour et al. 2014). This pattern has predominated since 2012, replacing the "ERRRKKR" pattern (Arafa et al. 2016a). All the isolates showed the R325K substitution at the cleavage site, which has been characteristic of 2.2.1.2 clade viruses since 2011 and is consistent with Arafa et al. (2016a). The diversity of the HA gene observed in the current study indicates the active circulation of the virus in the study area. This finding supports the genetic diversity of the H5N1 HA gene in Egypt (Arafa et al. 2016a).

Interestingly, Q322K substitution was obvious in four of our isolates compared to the reference and vaccine strains. This is indicative of continuous genetic evolution of the viruses. The higher similarity rate at the amino acid level among our isolates (98.8-100%) and higher identity between duck (98.8-100%), pigeon (97.7-100%) and quail (100%) when compared with the latest circulating isolates of each indicate the presence of a common ancestor progenitor. Similar

observations were previously reported in the same study area (ElBakrey et al. 2015). Egypt has three major risk factors that contribute to the endemicity of the disease: a high density of domestic waterfowl, high density of the rural human population and abundance of water and irrigation resources (Abdelwhab & Hafez 2011). The inadequate measures to control AIV in Egypt and the profound genetic drift in the HA gene have resulted in the endemic status of the disease (Kayali et al. 2016). Human cases of H5N1 were mostly caused by infection with viruses in subclade 2.2.1, which frequently circulate in LBMs and backyard poultry (Dudley 2009).

Molecular detection of H5N1 and H9N2 viruses in 89 respiratory patients revealed that 3.4% were infected with only the H5N1 subtype. These findings are in agreement with a recent study in Egypt, where the H5N1 subtype was reported in 5.8% of respiratory patients (Hussien et al. 2017). The low detection rate of HPAI H5N1 in human patients was consistent with other findings, thus supporting the previously reported low rate of transmission from birds to humans (Njabo et al. 2016). However, other studies have reported that none of the human participants in contact with infected birds were positive for the virus or antigens (Ghoneim et al. 2014). They attributed this to the need for mutations in the haemagglutination protein in humans for successful transmission from birds (Maines et al. 2011).

The results of our study indicated that the three H5N1 cases were in contact with poultry in rural areas, which is a predisposing factor for acquiring H5N1 infection; similar findings were previously reported (Hussien et al. 2017). The disease occurs more frequently in rural areas in the Nile Delta due to backyard rearing of birds and more frequent contact with birds sold in LBMs (Njabo et al. 2016). No specific symptoms were observed in the patients with H5N1 infection, which is consistent with other studies (CDC 2016; Hussien et al. 2017). Consequently, diagnosis of avian influenza infection cannot rely on clinical signs. The three positive cases in our study

were adult females (> 50 years). Kayali et al. (2011) reported that the case-fatality rate in humans infected with H5N1 was 34% and was significantly higher in female patients and increased with age. In contrast, the most affected demographic groups are young people and women (Njabo et al. 2016). Consistent with our results, recent H5N1 human cases reported exposure to poultry (70%), and most cases reported fever (98%), sore throat (94%) and cough (83%) (Kayali et al. 2016). The zoonotic potential of the H9N2 subtype and its co-existence with H5N1 have been documented in Egypt through occupational exposure (Gomaa et al. 2015). None of the human cases were positive for H9N2; however, a case was previously reported in South Egypt (OIE 2015). Continuous monitoring and surveillance is essential to prevent this subtype from infecting humans (Njabo et al. 2016).

Conclusions

The findings of this study indicated the circulation of endemic H5N1 and H9N2 viruses among poultry in 2015 and 2016. Birds on farms and in LBMs are reservoirs playing a role in the dissemination of the virus and producing a public health risk. Proper hygienic measures should be applied on farms and in LBMs to control the exposure of birds and humans to the source of infection. Continues surveillance and monitoring of the circulating viruses is important for understanding the evolution of the virus and to better select viruses for vaccine studies to minimize the wide spread of the viral infection.

Acknowledgements

The authors are thankful to Dr. Shimaa M. G. Mansour, assistant professor of virology and vet. Ahmed Magdy, assistant lecturer of zoonoses, Faculty of Veterinary Medicine, Zagazig University; for their valuable advice in analyzing the sequencing data.

References

Abdelwhab EM, Arafa AS, Stech J, Grund C, Stech O, Graeber-Gerberding M, Beer M, Hassan MK, Aly MM, Harder TC, and Hafez HM. 2012. Diversifying evolution of highly pathogenic H5N1 avian influenza virus in Egypt from 2006 to 2011. *Virus Genes* 45:14-23. 10.1007/s11262-012-0758-1

Abdelwhab EM, and Hafez HM. 2011. An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: epidemiology and control challenges. *Epidemiology and Infection* 139:647-657. 10.1017/s0950268810003122

Abdelwhab EM, Selim AA, Arafa A, Galal S, Kilany WH, Hassan MK, Aly MM, and Hafez MH. 2010. Circulation of avian influenza H5N1 in live bird markets in Egypt. *Avian Diseases* 54:911-914. 10.1637/9099-100809-resnote.1

Aguero M, Sanchez A, San Miguel E, Gomez-Tejedor C, and Jimenez-Clavero MA. 2007. A real-time TaqMan RT-PCR method for neuraminidase type 1 (N1) gene detection of H5N1 Eurasian strains of avian influenza virus. *Avian Diseases* 51:378-381. 10.1637/7642-050306r.1

Alexander DJ. 2007. An overview of the epidemiology of avian influenza. *Vaccine* 25:5637-5644. 10.1016/j.vaccine.2006.10.051

Amonsin A, Choatrakol C, Lapkuntod J, Tantilertcharoen R, Thanawongnuwech R, Suradhat S, Suwannakarn K, Theamboonlers A, and Poovorawan Y. 2008. Influenza Virus (H5N1) in Live Bird Markets and Food Markets, Thailand. *Emerging Infectious Diseases* 14:1739-1742. 10.3201/eid1411.080683

Arafa A, El-Masry I, Kholosy S, Hassan MK, Dauphin G, Lubroth J, and Makonnen YJ. 2016a. Phylodynamics of avian influenza clade 2.2.1 H5N1 viruses in Egypt. *Virology Journal* 13:49. 10.1186/s12985-016-0477-7

Arafa A, El-Masry I, Khoulosy S, Hassan MK, Soliman M, Fasanmi OG, Fasina FO, Dauphin G, Lubroth J, and Jobre YM. 2016b. Predominance and geo-mapping of avian influenza H5N1 in poultry sectors in Egypt. *Geospat Health* 11:492. 10.4081/gh.2016.492

Arafa A, Suarez D, Kholosy SG, Hassan MK, Nasef S, Selim A, Dauphin G, Kim M, Yilma J, Swayne D, and Aly MM. 2012a. Evolution of highly pathogenic avian influenza H5N1

viruses in Egypt indicating progressive adaptation. *Archives of Virology* 157:1931-1947. 10.1007/s00705-012-1385-9

Arafa AS, Hagag N, Erfan A, Mady W, El-Husseiny M, Adel A, and Nasef S. 2012b. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. *Virus Genes* 45:283-294. 10.1007/s11262-012-0775-0

Arafa AS, Hagag NM, Yehia N, Zanaty AM, Naguib MM, and Nasef SA. 2012c. Effect of cocirculation of highly pathogenic avian influenza H5N1 subtype with low pathogenic H9N2 subtype on the spread of infections. *Avian Diseases* 56:849-857. 10.1637/10152-040812-Reg.1

Ben Shabat M, Meir R, Haddas R, Lapin E, Shkoda I, Raibstein I, Perk S, and Davidson I. 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. *Journal of Virological Methods* 168:72-77. 10.1016/j.jviromet.2010.04.019

Cardona C, Yee K, and Carpenter T. 2009. Are live bird markets reservoirs of avian influenza? *Poultry Science* 88:856-859. 10.3382/ps.2008-00338

CDC. 2016. Center for disease control and prevention. Influenza signs and symptoms and the role of laboratory diagnostics. *http://wwwcdcgov/flu/professionals/diagnosis/labrolesprocedureshtm*.

Dudley JP. 2009. Age-specific infection and death rates for human A(H5N1) avian influenza in Egypt. *Euro Surveillance* 14.

El-Zoghby EF, Aly MM, Nasef SA, Hassan MK, Arafa AS, Selim AA, Kholousy SG, Kilany WH, Safwat M, Abdelwhab EM, and Hafez HM. 2013. Surveillance on A/H5N1 virus in domestic poultry and wild birds in Egypt. *Virology Journal* 10:203. 10.1186/1743-422x-10-203

El-Zoghby EF, Arafa AS, Hassan MK, Aly MM, Selim A, Kilany WH, Selim U, Nasef S, Aggor MG, Abdelwhab EM, and Hafez HM. 2012. Isolation of H9N2 avian influenza virus from bobwhite quail (Colinus virginianus) in Egypt. *Archives of Virology* 157:1167-1172. 10.1007/s00705-012-1269-z

El Houadfi M, Fellahi S, Nassik S, Guérin J-L, and Ducatez MF. 2016. First outbreaks and phylogenetic analyses of avian influenza H9N2 viruses isolated from poultry flocks in Morocco. *Virology Journal* 13:140. 10.1186/s12985-016-0596-1

ElBakrey RM, El Sisi MA, Mansour SM, Ahmed HH, Rajput M, and Eid AA. 2015. Cleavage site stability of Egyptian highly pathogenic avian influenza viruses in backyard chickens during 2009-2011. *Journal of Microbiology, Immunology, and Infection Wei Mian Yu Gan Ran Za Zhi* 48:28-35. 10.1016/j.jmii.2013.12.002

Elgendy EM, Watanabe Y, Daidoji T, Arai Y, Ikuta K, Ibrahim MS, and Nakaya T. 2016. Genetic characterization of highly pathogenic avian influenza H5N1 viruses isolated from naturally infected pigeons in Egypt. *Virus Genes* 52:867-871. 10.1007/s11262-016-1369-z

ElMasry I, Elshiekh H, Abdlenabi A, Saad A, Arafa A, Fasina FO, Lubroth J, and Jobre YM. 2017. Avian Influenza H5N1 Surveillance and its Dynamics in Poultry in Live Bird Markets, Egypt. *Transboundary and Emerging Diseases* 64:805-814. 10.1111/tbed.12440

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, and Osterhaus AD. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *Journal of Virology* 79:2814-2822. 10.1128/jvi.79.5.2814-2822.2005

Ghoneim NH, Abdel-Moein KA-A, and Zaher HM. 2014. Epidemiology of avian influenza H5N1 virus in Egypt and its zoonotic potential. *Asian Pacific Journal of Tropical Disease* 4:S631-S634. https://doi.org/10.1016/S2222-1808(14)60693-9

Gomaa MR, Kayed AS, Elabd MA, Zeid DA, Zaki SA, El Rifay AS, Sherif LS, McKenzie PP, Webster RG, Webby RJ, Ali MA, and Kayali G. 2015. Avian influenza A(H5N1) and A(H9N2) seroprevalence and risk factors for infection among Egyptians: a prospective, controlled seroepidemiological study. *Journal of Infectious Diseases* 211:1399-1407. 10.1093/infdis/jiu529

Greenacre M. 2017. *Correspondence Analysis in Practice*. Chapmann & Hall/CRC: Boca Raton.

Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, Norwood M, Shortridge KF, Webster RG, and Guan Y. 2000. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology* 267:279-288. 10.1006/viro.1999.0115

Hafez MH, Arafa A, Abdelwhab EM, Selim A, Khoulosy SG, Hassan MK, and Aly MM. 2010. Avian influenza H5N1 virus infections in vaccinated commercial and backyard poultry in Egypt. *Poultry Science* 89:1609-1613. 10.3382/ps.2010-00708

Hagag IT, Mansour SMG, Zhang Z, Ali AAH, Ismaiel E-BM, Salama AA, Cardona CJ, Collins J, and Xing Z. 2015. Pathogenicity of Highly Pathogenic Avian Influenza Virus H5N1 in Naturally Infected Poultry in Egypt. *PloS One* 10:e0120061. 10.1371/journal.pone.0120061

Hassan MK, Jobre Y, Arafa A, Abdelwhab EM, Kilany WH, Khoulosy SG, Bakry NR, Baile E, Ali A, Ankers P, and Lubroth J. 2013. Detection of A/H5N1 virus from asymptomatic native ducks in mid-summer in Egypt. *Archives of Virology* 158:1361-1365. 10.1007/s00705-012-1599-x

Hussien AA, Hassanien AA, Salama S, Ewida R, and Awad M. 2017. Investigation of the emergence of avian and swine influenza among respiratory patients in Assiut University Hospital, Egypt. *Pakistan Veterinary Journal* 37:220-224.

Ibrahim AA, Albrechtsen L, Upton M, Morgan N, and Rushton J. 2007. Market Impacts of HPAI Outbreaks: A Rapid Appraisal Process, Egypt 2007. Report submitted to FAO 2007. p 33.

Jia B, Shi J, Li Y, Shinya K, Muramoto Y, Zeng X, Tian G, Kawaoka Y, and Chen H. 2008. Pathogenicity of Chinese H5N1 highly pathogenic avian influenza viruses in pigeons. *Archives of Virology* 153:1821-1826. 10.1007/s00705-008-0193-8

Joannis TM, Meseko CA, Oladokun AT, Ularamu HG, Egbuji AN, Solomon P, Nyam DC, Gado DA, Luka P, Ogedengbe ME, Yakubu MB, Tyem AD, Akinyede O, Shittu AI, Sulaiman LK, Owolodun OA, Olawuyi AK, Obishakin ET, and Fasina FO. 2008. Serologic and virologic surveillance of avian influenza in Nigeria, 2006-7. *Eurosurveillance* 13:19007. doi:https://doi.org/10.2807/ese.13.42.19007-en

Kalthoff D, Globig A, and Beer M. 2010. (Highly pathogenic) avian influenza as a zoonotic agent. *Veterinary Microbiology* 140:237-245. 10.1016/j.vetmic.2009.08.022

Kandeil A, El-Shesheny R, Maatouq A, Moatasim Y, Cai Z, McKenzie P, Webby R, Kayali G, and Ali MA. 2017. Novel reassortant H9N2 viruses in pigeons and evidence for antigenic diversity of H9N2 viruses isolated from quails in Egypt. *Journal of General Virology* 98:548-562. 10.1099/jgv.0.000657

Kayali G, El-Shesheny R, Kutkat MA, Kandeil AM, Mostafa A, Ducatez MF, McKenzie PP, Govorkova EA, Nasraa MH, Webster RG, Webby RJ, and Ali MA. 2011. Continuing threat of influenza (H5N1) virus circulation in Egypt. *Emerging Infectious Diseases* 17:2306-2308. 10.3201/eid1712.110683

Kayali G, Kandeil A, El-Shesheny R, Kayed AS, Gomaa MM, Maatouq AM, Shehata MM, Moatasim Y, Bagato O, Cai Z, Rubrum A, Kutkat MA, McKenzie PP, Webster RG, Webby RJ, and Ali MA. 2014. Active surveillance for avian influenza virus, Egypt, 2010-2012. *Emerging Infectious Diseases* 20:542-551. 10.3201/eid2004.131295

Kayali G, Kandeil A, El-Shesheny R, Kayed AS, Maatouq AM, Cai Z, McKenzie PP, Webby RJ, El Refaey S, Kandeel A, and Ali MA. 2016. Avian Influenza A(H5N1) Virus in Egypt. *Emerging Infectious Diseases* 22:379-388. 10.3201/eid2203.150593

Klopfleisch R, Werner O, Mundt E, Harder T, and Teifke JP. 2006. Neurotropism of highly pathogenic avian influenza virus A/chicken/Indonesia/2003 (H5N1) in experimentally infected pigeons (Columbia livia f. domestica). *Veterinary Pathology* 43:463-470. 10.1354/vp.43-4-463

Londt BZ, Nunez A, Banks J, Nili H, Johnson LK, and Alexander DJ. 2008. Pathogenesis of highly pathogenic avian influenza A/turkey/Turkey/1/2005 H5N1 in Pekin ducks (Anas

564 platyrhynchos) infected experimentally. *Avian Pathology* 37:619-627.
565 10.1080/03079450802499126
566
567 Mady WH, El-Sanousi AA, Amer HM, El-Sabagh IM, Khafagy AM, and Saber MS. 2010. First
568 year of the Highly Pathogenic Avian Influenza H5N1 outbreak in Egypt: rapid
569 antigenic/molecular diagnosis and virus isolation. *International Journal of Virology* 6:73-
570 81.
571
572 Maines TR, Chen LM, Van Hoeven N, Tumpey TM, Blixt O, Belser JA, Gustin KM, Pearce MB,
573 Pappas C, Stevens J, Cox NJ, Paulson JC, Raman R, Sasisekharan R, Katz JM, and Donis
574 RO. 2011. Effect of receptor binding domain mutations on receptor binding and
575 transmissibility of avian influenza H5N1 viruses. *Virology* 413:139-147.
576 10.1016/j.virol.2011.02.015
577
578 Mansour SM, ElBakrey RM, Ali H, Knudsen DE, and Eid AA. 2014. Natural infection with highly
579 pathogenic avian influenza virus H5N1 in domestic pigeons (Columba livia) in Egypt.
580 *Avian Pathology* 43:319-324. 10.1080/03079457.2014.926002
581
582 Mansour SMG, Mohamed FF, Eid AAM, Mor SK, and Goyal SM. 2017. Co-circulation of
583 paramyxo- and influenza viruses in pigeons in Egypt. *Avian Pathology* 46:367-375.
584 10.1080/03079457.2017.1285391
585
586 Naeem K, Ullah A, Manvell RJ, and Alexander DJ. 1999. Avian influenza A subtype H9N2 in
587 poultry in Pakistan. *Veterinary Record* 145:560.
588
589 Naguib MM, Arafa AS, El-Kady MF, Selim AA, Gunalan V, Maurer-Stroh S, Goller KV, Hassan
590 MK, Beer M, Abdelwhab EM, and Harder TC. 2015. Evolutionary trajectories and
591 diagnostic challenges of potentially zoonotic avian influenza viruses H5N1 and H9N2 co-
592 circulating in Egypt. *Infection, Genetics and Evolution* 34:278-291.
593 10.1016/j.meegid.2015.06.004
594
595 Nguyen DC, Uyeki TM, Jadhao S, Maines T, Shaw M, Matsuoka Y, Smith C, Rowe T, Lu X, Hall
596 H, Xu X, Balish A, Klimov A, Tumpey TM, Swayne DE, Huynh LP, Nghiem HK, Nguyen
597 HH, Hoang LT, Cox NJ, and Katz JM. 2005. Isolation and characterization of avian
598 influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in
599 Hanoi, Vietnam, in 2001. *Journal of Virology* 79:4201-4212. 10.1128/jvi.79.7.4201-
600 4212.2005
601
602 Nguyen TH, Than VT, Thanh HD, Hung V-K, Nguyen DT, and Kim W. 2016. Intersubtype
603 Reassortments of H5N1 Highly Pathogenic Avian Influenza Viruses Isolated from Quail.
604 *PloS One* 11:e0149608. 10.1371/journal.pone.0149608
605
606 Njabo KY, Zanontian L, Sheta BN, Samy A, Galal S, Schoenberg FP, and Smith TB. 2016. Living
607 with avian FLU--Persistence of the H5N1 highly pathogenic avian influenza virus in Egypt.
608 *Veterinary Microbiology* 187:82-92. 10.1016/j.vetmic.2016.03.009
609

OIE. 2012. Manual of diagnostic tests and vaccines for terrestrial animals (Chapter 2.3.4). *http://wwwoieint/en/international-standard-setting/terrestrial-manual/access-online/ accessed on 25 May 2013*.

OIE. 2015. World Organization for Animal Health (OIE) manual on animal influenza diagnosis and virological isolation. *http://wwwoieint/fileadmin/Home/fr/Health_standards/tahm/20304_AIpdf*.

Rhyoo MY, Lee KH, Moon OK, Park WH, Bae YC, Jung JY, Yoon SS, Kim HR, Lee MH, Lee EJ, Ki MR, and Jeong KS. 2015. Analysis of signs and pathology of H5N1-infected ducks from the 2010-2011 Korean highly pathogenic avian influenza outbreak suggests the influence of age and management practices on severity of disease. *Avian Pathology* 44:175-181. 10.1080/03079457.2015.1021295

Slomka MJ, Coward VJ, Banks J, Londt BZ, Brown IH, Voermans J, Koch G, Handberg KJ, Jorgensen PH, Cherbonnel-Pansart M, Jestin V, Cattoli G, Capua I, Ejdersund A, Thoren P, and Czifra G. 2007. Identification of sensitive and specific avian influenza polymerase chain reaction methods through blind ring trials organized in the European Union. *Avian Diseases* 51:227-234. 10.1637/7674-063006r1.1

Smietanka K, Minta Z, Wyrostek K, Jozwiak M, Olszewska M, Domanska-Blicharz AK, Reichert AM, Pikula A, Habyarimana A, and van den Berg T. 2011. Susceptibility of pigeons to clade 1 and 2.2 high pathogenicity avian influenza H5N1 virus. *Avian Diseases* 55:106-112. 10.1637/9514-090110-ResNote.1

Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, and Poovorawan Y. 2006. Avian Influenza H5N1 in Naturally Infected Domestic Cat. *Emerging Infectious Diseases* 12:681-683. 10.3201/eid1204.051396

Sun Y, Qin K, Wang J, Pu J, Tang Q, Hu Y, Bi Y, Zhao X, Yang H, Shu Y, and Liu J. 2011. High genetic compatibility and increased pathogenicity of reassortants derived from avian H9N2 and pandemic H1N1/2009 influenza viruses. *Proceedings of the National Academy of Sciences of the United States of America* 108:4164-4169. 10.1073/pnas.1019109108

Swayne DE, and Suarez DL. 2000. Highly pathogenic avian influenza. *Revue Scientifique et Technique* 19:463-482.

Thontiravong A, Kitikoon P, Wannaratana S, Tantilertcharoen R, Tuanudom R, Pakpinyo S, Sasipreeyajan J, Oraveerakul K, and Amonsin A. 2012. Quail as a potential mixing vessel for the generation of new reassortant influenza A viruses. *Veterinary Microbiology* 160:305-313. 10.1016/j.vetmic.2012.05.043

Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, and Donis RO. 2012. A distinct lineage of influenza A virus from bats. *Proceedings of the*

National Academy of Sciences of the United States of America 109:4269-4274. 10.1073/pnas.1116200109

Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, and Donis RO. 2013. New world bats harbor diverse influenza A viruses. *PLoS Pathogens* 9:e1003657. 10.1371/journal.ppat.1003657

Turner JC, Feeroz MM, Hasan MK, Akhtar S, Walker D, Seiler P, Barman S, Franks J, Jones-Engel L, McKenzie P, Krauss S, Webby RJ, Kayali G, and Webster RG. 2017. Insight into live bird markets of Bangladesh: an overview of the dynamics of transmission of H5N1 and H9N2 avian influenza viruses. *Emerg Microbes Infect* 6:e12. 10.1038/emi.2016.142

Wang M, Di B, Zhou DH, Zheng BJ, Jing H, Lin YP, Liu YF, Wu XW, Qin PZ, Wang YL, Jian LY, Li XZ, Xu JX, Lu EJ, Li TG, and Xu J. 2006. Food markets with live birds as source of avian influenza. *Emerging Infectious Diseases* 12:1773-1775. 10.3201/eid1211.060675

WHO. 2006. Epidemiology of WHO-confirmed human cases of avian influenza A(H5N1) infection. *Weekly Epidemiological Record* 81:249-257.

WHO. 2017. Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003-2016. *http://wwwwhoint/influenza/human_animal_interface/2016_11_21_tableH5N1pdf?ua=1*.

Woo GH, Kim HY, Bae YC, Jean YH, Bak EJ, Kim MJ, Hwang EK, and Joo YS. 2011. Comparative histopathological characteristics of highly pathogenic avian influenza (HPAI) in chickens and domestic ducks in 2008 Korea. *Histology and Histopathology* 26:167-175. 10.14670/hh-26.167

Zahida F, Muhammad AK, Mansur-ud-Din A, Khushi M, Khalid NK, Amjad K, Zubair A, Abdul A, and Altaf M. 2017. Cross sectional survey of live bird markets and zoo birds for circulating influenza subtypes in Pakistan. *Pakistan Veterinary Journal* 37:185-189.

Zecchin B, Minoungou G, Fusaro A, Moctar S, Ouedraogo-Kaboré A, Schivo A, Salviato A, Marciano S, and Monne I. 2017. Influenza A(H9N2) Virus, Burkina Faso. *Emerging Infectious Diseases* 23:2118-2119. 10.3201/eid2312.171294

Table 1(on next page)

Proportion of positive samples for H5N1 and H9N2 subtypes determined by RT-PCR.

Table 1. Proportion of positive samples for H5N1 and H9N2 subtypes determined by RT-PCR.

Birds	Farms			LBMs		
	Number examined	H5N1 subtype	H9N2 subtype	Number examined	H5N1 subtype	H9N2 subtype
Ducks	24	4 (16.7%)	0 (0%)	45	9 (20%)	0 (0%)
Pigeons	31	4 (12.9%)	2 (6.5%)	35	7 (20%)	4 (11.4%)
Quails	4	0 (0%)	0 (0%)	20	1 (5%)	0 (0%)
Total	**59**	**8 (13.6%)**	**2 (3.4%)**	**100**	**17 (17%)**	**4 (4%)**

Table 2(on next page)

Demographic, Knowledge, attitudes and practices data of 89 respiratory patients screened for H5N1 and H9N2 virus infection with real-time RT-PCR.

Table 2. Demographic, Knowledge, attitudes and practices data of 89 respiratory patients screened for H5N1 and H9N2 virus infection with real-time RT-PCR.

Variables	Collected samples, no. (%)	Influenza A-positive samples, no. (%)	*P*-value
I. Demographic characteristics			
Gender			
Male	33 (37.1%)	0 (0%)	0.292
Female	56 (62.9%)	3 (5.4%)	
Age			
≤19 years	25 (28.1%)	0 (0%)	0.253
20-49 years	25 (28.1%)	0 (0%)	
≥50 years	39 (43.8%)	3 (7.7%)	
Educational level			
Illiterate	30 (33.7%)	3 (10%)	0.389
Primary	9 (10.1%)	0 (0%)	
Secondary	17 (19.1%)	0 (0%)	
High school	15 (16.8%)	0 (0%)	
University	18 (20.2%)	0 (0%)	
Residence			
Rural	50 (56.2%)	3 (6%)	0.173
Urban	39 (43.8%)	0 (0%)	
Occupations			
Employer	24 (27%)	0 (0%)	0.163
Farmer	11 (12.4%)	0 (0%)	
Household	31 (34.8%)	3 (9.7%)	
Student	23 (25.8%)	0 (0%)	
II. Knowledge			
Had heard of AIV			
Yes	75 (84.3%)	3 (4%)	1.00
No	14 (15.7%)	0 (0%)	
AIV is an infectious to human			
Yes	50 (56.2%)	1 (2%)	0.264
No	7 (7.9%)	1 (14.3%)	
Don't know	32 (35.9%)	1 (3.1%)	
Information source			
Television	58 (65.2%)	3 (5.2%)	1.00
Internet	17 (19.1%)	0 (0%)	
None	14 (15.7%)	0 (0%)	
Knowledge about modes of AIV transmission			
Yes	46 (51.7%)	0 (0%)	0.109
No	43 (48.3%)	3 (7%)	
Knowledge about AIV symptoms in human			
Yes	46 (51.7%)	0 (0%)	0.109
No	43 (48.3%)	3 (7%)	
III. Attitudes and Practices			
Direct contact with to poultry			
Yes	56 (62.9%)	3 (5.4%)	0.244
No	33 (37.1%)	0 (5.4%)	
Use of preventive measures during contact with poultry			
Yes	9 (10.1%)	0 (0%)	0.723
No	80 (89.9%)	3 (3.8%)	
Healthcare-seeking behaviour			
Purchasing cold medicine by myself	62 (69.7%)	3 (4.8%)	0.550
Seeking medical service from hospitals	27 (30.3%)	0 (0%)	
Influenza vaccine administration			
Yes	33 (37.1%)	0 (0%)	0.244
No	56 (62.9%)	3 (5.4%)	
Total	**89**	**3 (3.4%)**	

3

Figure 1

Clinical manifestations and PM lesions of birds suspected to be infected with AIVs.

(A) nervous signs (twisting of neck) in 3 months old pigeon, (B) necrosis in pancreas of two months old Balady duck, (C) congestion with petechial hemorrhage in the brain of 1.5 months old Muscovy duck, (D) congestion in spleen of 1.5 months old Muscovy duck, (E) congestion in the brain of 3 months old pigeon with petechial hemorrhage in the inner aspect of the skull, (F) congestion in the liver of 1 year old Balady pigeon.

Photo credit: Hala Tolba.

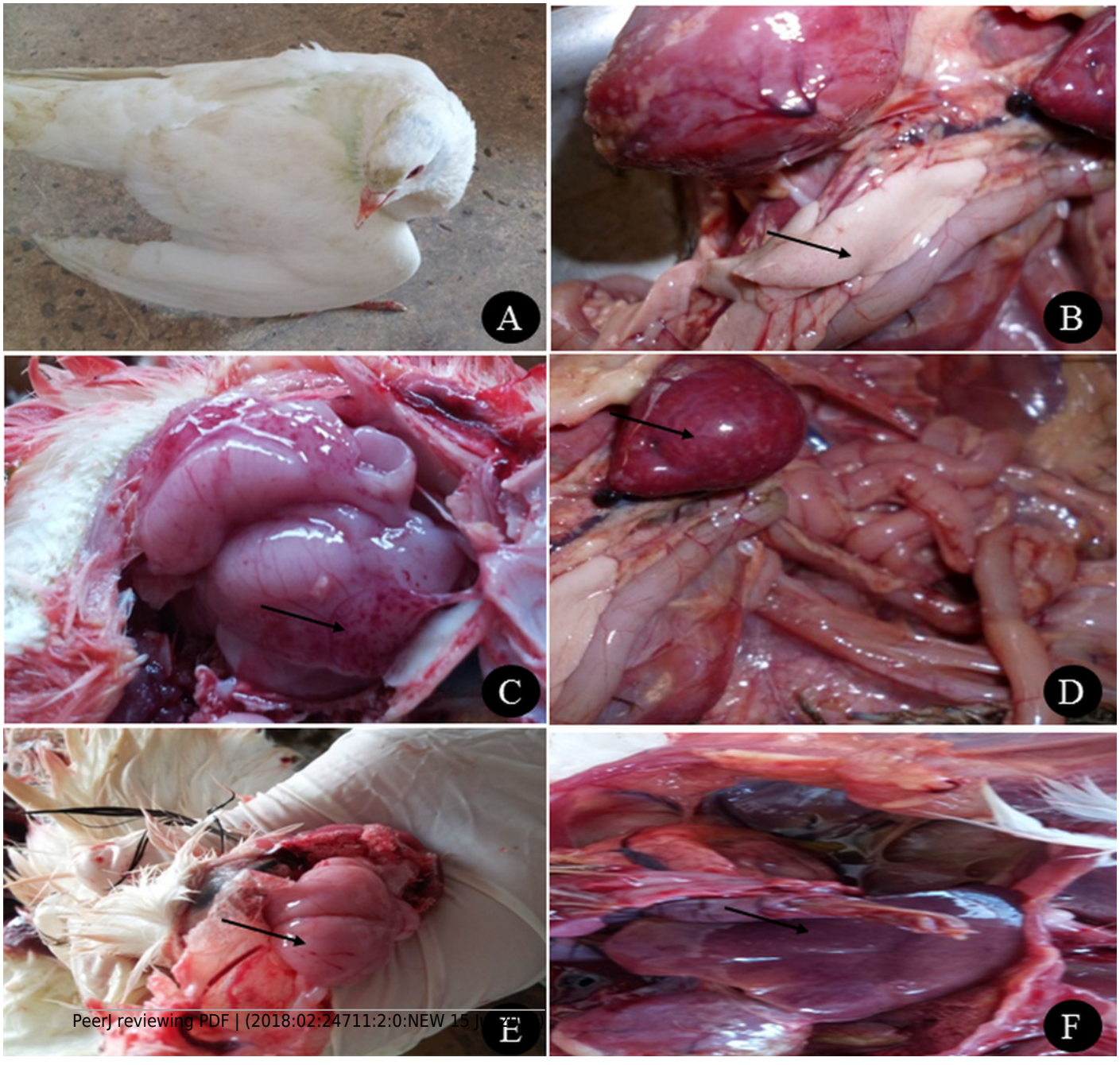


Figure 2

Deduced amino acid sequences of the HA protein

Deduced amino acid sequences of the HA protein of our isolates (isolates from farms are marked by an asterisk) in comparison to parent (A/chicken/Egypt/06207-NLQP/2006), classic (A/chicken/Egypt/NLQP-0918/2009), variant (A/chicken/Egypt/0879/2008), vaccinal H5N1 (A/chicken/Egypt/Q1995D/2010), H5N2 (A/duck/Potsdam/1402-6/1986) strains and latest duck in Sharkia (A/duck/Egypt/1435CAS/2014), latest duck in Egypt (A/duck/Egypt/2/2015), latest pigeon in Sharkia (A/pigeon/Egypt/Sharkia-22/2014) and latest quail strains in Egypt (A/quail/Egypt/14102TCP/2014 and A/quail/Egypt/1171SG/2011). Dots denote identical amino acids. The H5 influenza numbering was based on the alignment with A/Goose/Guangdong/1/96 (H5N1) minus the 16 amino acids known as HA signal peptide.

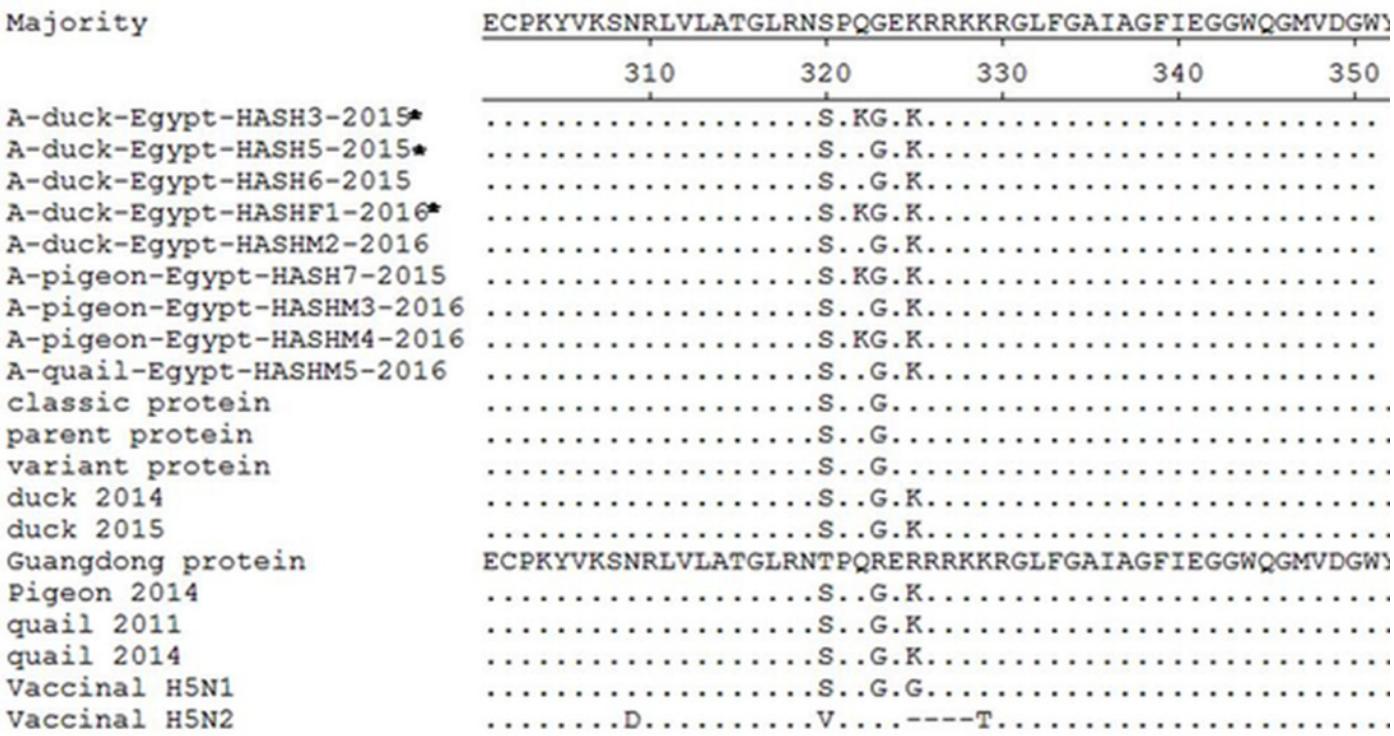


Figure 3

Phylogenetic analysis of HA gene nucleotide sequences of avian influenza virus (AIV) isolated from ducks, pigeons and quail, Sharkia, Egypt and other sequences available in GenBank.

The strains isolated in winter 2015 are marked by solid circles, while those of winter 2016 are marked with solid squares and vaccinal strains are marked with solid triangles. The tree was constructed via multiple alignments of 300-bp nucleotide sequence of HA gene using the neighbor-joining method and the Kimura-2-parameter model in MEGA7. The tree topology was evaluated by 1000 bootstrap analyses.

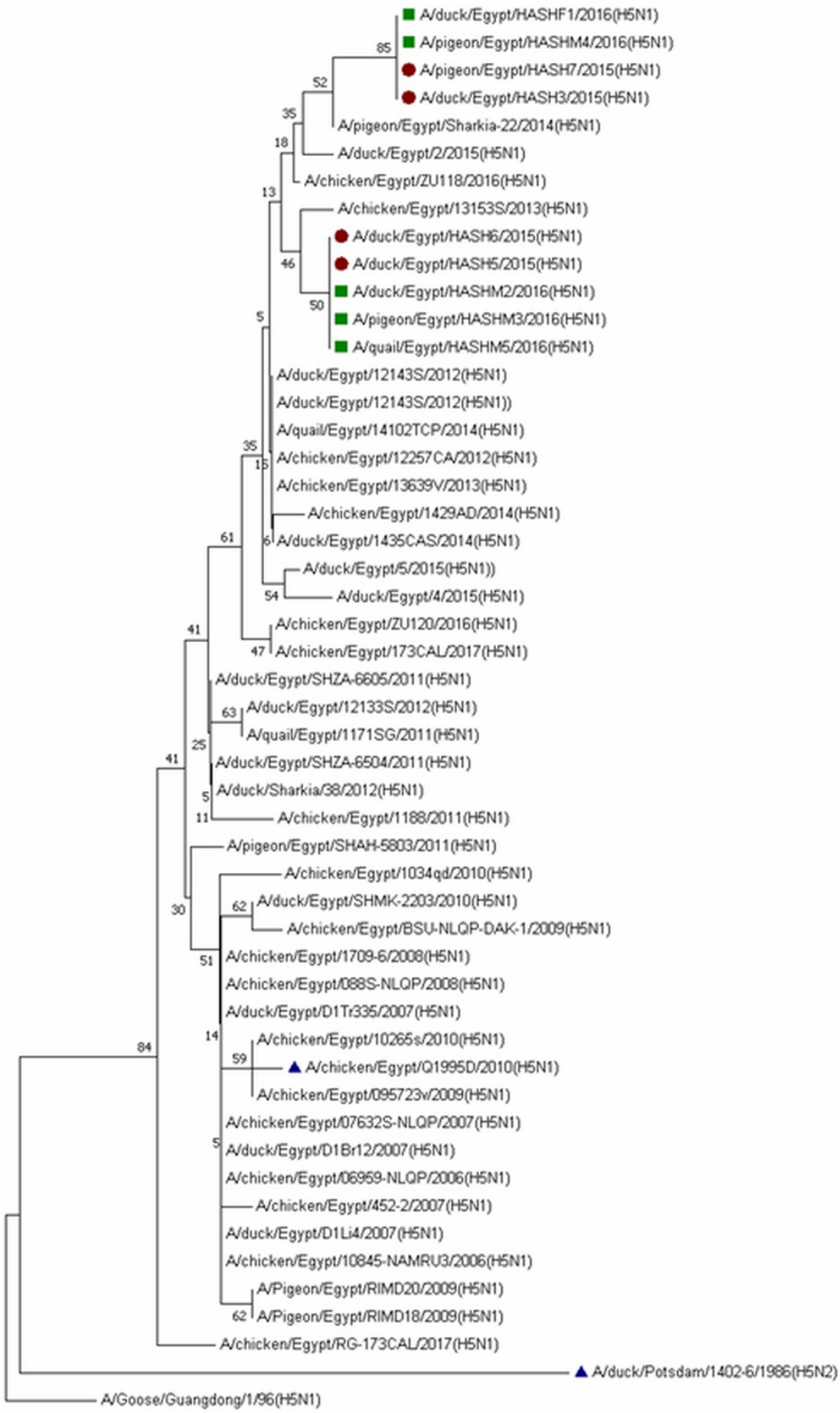
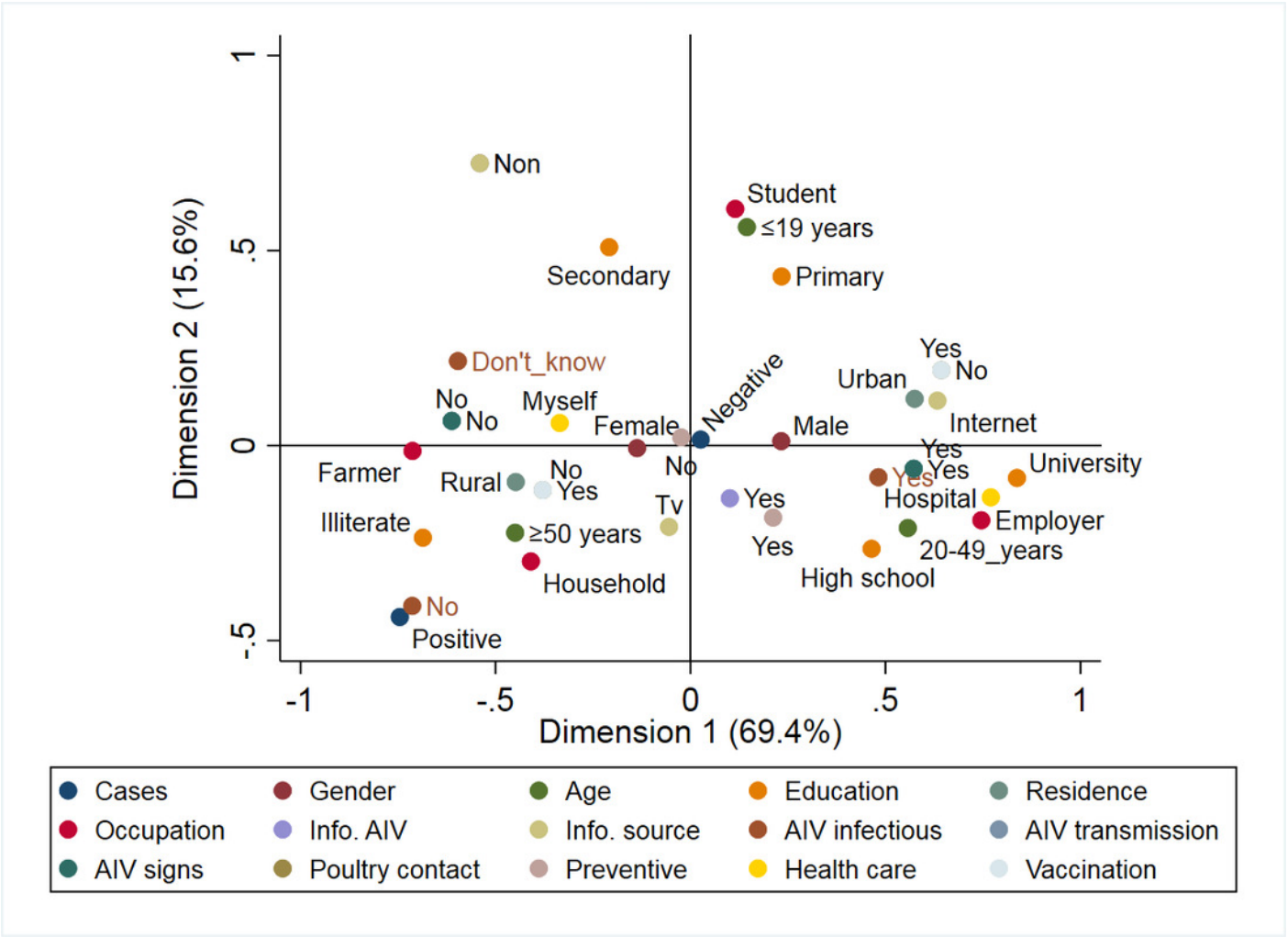

A/duck/Egypt/HASHF1/2016(H5N1)
A/pigeon/Egypt/HASHM4/2016(H5N1)
A/pigeon/Egypt/HASH7/2015(H5N1)
A/duck/Egypt/HASH3/2015(H5N1)
A/pigeon/Egypt/Sharkia-22/2014(H5N1)
A/duck/Egypt/2/2015(H5N1)
A/chicken/Egypt/ZU118/2016(H5N1)
A/chicken/Egypt/13153S/2013(H5N1)
A/duck/Egypt/HASH6/2015(H5N1)
A/duck/Egypt/HASH5/2015(H5N1)
A/duck/Egypt/HASHM2/2016(H5N1)
A/pigeon/Egypt/HASHM3/2016(H5N1)
A/quail/Egypt/HASHM5/2016(H5N1)
A/duck/Egypt/12143S/2012(H5N1)
A/duck/Egypt/12143S/2012(H5N1))
A/quail/Egypt/14102TCP/2014(H5N1)
A/chicken/Egypt/12257CA/2012(H5N1)
A/chicken/Egypt/13639V/2013(H5N1)
A/chicken/Egypt/1429AD/2014(H5N1)
A/duck/Egypt/1435CAS/2014(H5N1)
A/duck/Egypt/5/2015(H5N1))
A/duck/Egypt/4/2015(H5N1)
A/chicken/Egypt/ZU120/2016(H5N1)
A/chicken/Egypt/173CAL/2017(H5N1)
A/duck/Egypt/SHZA-6605/2011(H5N1)
A/duck/Egypt/12133S/2012(H5N1)
A/quail/Egypt/1171SG/2011(H5N1)
A/duck/Egypt/SHZA-6504/2011(H5N1)
A/duck/Sharkia/38/2012(H5N1)
A/chicken/Egypt/1188/2011(H5N1)
A/pigeon/Egypt/SHAH-5803/2011(H5N1)
A/chicken/Egypt/1034qd/2010(H5N1)
A/duck/Egypt/SHMK-2203/2010(H5N1)
A/chicken/Egypt/BSU-NLQP-DAK-1/2009(H5N1)
A/chicken/Egypt/1709-6/2008(H5N1)
A/chicken/Egypt/088S-NLQP/2008(H5N1)
A/duck/Egypt/D1Tr335/2007(H5N1)
A/chicken/Egypt/10265s/2010(H5N1)
A/chicken/Egypt/Q1995D/2010(H5N1)
A/chicken/Egypt/095723w/2009(H5N1)
A/chicken/Egypt/07632S-NLQP/2007(H5N1)
A/duck/Egypt/D1Br12/2007(H5N1)
A/chicken/Egypt/06959-NLQP/2006(H5N1)
A/chicken/Egypt/452-2/2007(H5N1)
A/duck/Egypt/D1Li4/2007(H5N1)
A/chicken/Egypt/10845-NAMRU3/2006(H5N1)
A/Pigeon/Egypt/RIMD20/2009(H5N1)
A/Pigeon/Egypt/RIMD18/2009(H5N1)
A/chicken/Egypt/RG-173CAL/2017(H5N1)
A/duck/Potsdam/1402-6/1986(H5N2)
A/Goose/Guangdong/1/96(H5N1)
0.02

Figure 4

Multiple correspondence analysis of risk factors associated with the avian influenza virus (AIV) infections in respiratory patients admitted to the general hospitals in Sharkia Governates.