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Full Length Article

# Co-circulation of avian influenza viruses in commercial farms, backyards and live market birds in Egypt


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**Abstract** Cloacal and tracheal swab-samples were collected from commercial farms, backyards and live market birds (LBM) to identify the potential existence and genetic drifts of avian influenza subtypes (AI) H5 and H9 that are circulating among bird species in Egypt. The results revealed that, one sample out of 50 samples of chicken commercial farms was positive for the isolation of subtype H9N2 [KC699549, Influenza A virus: A/chicken/Egypt/VRLCU-R33/2012(H9N2)]; from Sharkeia province. Two samples out of 20 samples of Backyard ducks were positive for the isolation of 2 subtypes H5N1; [KC699547, Influenza A virus: A/duck/Egypt/VRLCU-R11/2012(H5N1), "backyard duck"] from El-Fayoum province and the other from Giza province [A/duck/Egypt/VRLCU-R28/2012(H5N1), "backyard duck"]. Analysis of haemagglutinin (HA) and the phylogenetic tree of the isolated viruses (H5N1) were fallen within the clade 2.2.1.1. Antigenic cartography for the isolated Egyptian H9N2 AI virus can intuitively be of group-B. The number of mutations in the amino acid sites (33, 47, 65, 90, 92, 143, and 150) and the Long Branch observed in the phylogenetic tree may suggest a rather long evolution period. The sequenced H9N2 Egyptian virus in the study was closely related to the previous Egyptian isolates.



## 1. Introduction

Avian influenza (AI) is a highly contagious respiratory disease affecting poultry caused by influenza A viruses of the family Orthomyxoviridae. Influenza A viruses are classified into 17 haemagglutinin (HA) subtypes and 10 neuraminidase subtypes

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[1]. The disease constitutes a major threat to poultry industry worldwide [2], where a high number of birds involved the extreme control costs of the disease and the gregarious consequences [3].

In 1996, A/goose/Guangdong/1/1996(H5N1), was identified in a geese farm in southern China. These H5N1 HPAIVs have been spread across Asia, Europe and Africa and presented a continuous threat to both animal and human health [4].

Highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype descending from A/goose/Guangdong/1/96 lineage was first detected in Africa in 2006 [5].

In March 2006, vaccination of poultry using inactivated vaccines derived from either Mexican low pathogenic H5N2 or Asian H5N1 strains was sanctioned essentially [6].

In 2008, H5N1 HPAI infection perpetuated to fan out and became endemic in Egypt. Clade 2.2.1 was introduced into Egypt and spread rapidly in commercial and backyard flocks [7]. As a consequence of the persistence and extensive circulation of H5N1 HPAI viruses in Egypt, variant strains emerged evolving into distinct genetic subclades [8].

One of these variant strains is H9N2 AIVs which have circulated worldwide in poultry populations over the last decade, causing mild respiratory disease and reductions in egg production, resulting in great economic losses and co-infection with other pathogens [9–15].

H9 subtypes posses a mild nature which may provide them a great opportunity to turn more virulent through surreptitious spread, mutation and/or reassortment with other subtypes of influenza viruses [16–18].

Antigenic drift has been observed in type A influenza viruses resulting from point mutations which ultimately transmute the hemagglutinin (HA) protein epitope structure [19].

This study aimed to identify the potential existence and antigenic drift of Avian Influenza subtypes (AI) H5 and H9 circulating among chicken flocks, backyards and live bird markets in Egypt.

## 2. Materials and Methods

### 2.1. Samples

Cloacal and tracheal swab-samples were amassed from 50 Commercial farms (broilers, layers, breeders), 10 Backyard and LBM bird species (ducks, geese, turkeys, chickens) during 2012 in some Egyptian provinces [20].

### 2.2. Virus isolation, RT-PCR, sequencing and data analysis

#### 2.2.1. Virus isolation

Specific pathogen free embryonated chicken eggs (ECE) of 9–11 day old were used for isolation and propagation of the avian viruses. The eggs were obtained from SPF engenderment project, Fayoum, Egypt.

#### 2.2.2. Viral RNAs extraction

Viral RNAs were extracted by the use of QIAamp viral RNA Mini Kit (QIAGEN, Germany) Cat. No. 52904. The kit combines the selective binding properties of silica-gel-based membrane with the speed of micro spin technology. The kit contains: QIAamp mini spin columns, collection tubes (2 ml), buffer (AVL), buffer AW1, buffer AW2, and buffer AVE and Carrier RNA.

#### 2.2.3. Amplification

Primers were designed for specific help in genetic characterization of AI strain. The design of primers was according to lab of Virology, Faculty of Veterinary Medicine, Cairo University and was manufactured by METABION® Company (Germany).

#### 2.2.4. Sequencing

One-Step RT-PCR kit (QIAGEN, Germany) with primers specific for influenza virus. Cat. No. 210212, was used. The primer sequences and amplification conditions used were available upon request. The PCR products were separated on an agarose gel (Vivantis-Malaysia) by electrophoresis, and amplicons of the felicitous sizes were subsequently excised from the gel and extracted by use of a QIAGEN gel extraction kit.

#### 2.2.5. Phylogenetic analysis of influenza virus genes

Phylogenetic and molecular evolutionary analyses were conducted using BIOEDIT version 7.0.4.1 (MEGA 5.05 software) (95/98/NT/2000/XP). MEGA is an integrated implement for conducting automatic and manual sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. MEGA is a multi-threaded windows application. It runs on all releases of Microsoft Windows operating system. Sequence submission to the Gen-bank (Sequence FASTA file): The frame adjusted, clean sequence which used in phylogenetic tree construction was submitted to Gen-bank. Sequence results were received via mail as text, BLAST report and AB1 file for both forward and reverse sequence of the sent sample.

#### 2.2.6. Deduced amino acid sequence analysis

We analyzed the HA deduced amino acid sequences of 3 isolated strains and compared them with Egyptian H5N1 and H9N2 isolates available in the flu database.

## 3. Results

One sample out of 50 samples (2%) of chicken commercial farms was positive for the isolation of one subtype of H9N2; from Sharkeia province. Two samples out of 20 samples (10%) of Backyard species were positive for the isolation of 2 subtypes of H5N1; from ducks, one from El-Fayoum province and the other from Giza province, as indicated in Table 1.

**Table 1** Frequency of virus detection by RT-PCR in commercial farms, backyard species and LBM.

| Province | RT-PCR commercial farms | | RT-PCR backyard and LBM[1] | |
|---|---|---|---|---|
| | No. | % | No. | % |
| Sharkeia | 1\12[2] | 4.8 | 0 | 0 |
| Dakahlya | 0\10 | 0 | 0 | 0 |
| Kaleobia | 1\8 | 12.5 | – | – |
| EL-Behera | 0\10 | 0 | 0 | 0 |
| El-Fayoum[3] | – | – | 1\10 | 20 |
| Giza[4] | – | – | 1\10 | 20 |
| Total | 1\50 | 2 | 2\20 | 10 |

[1] Ducks, geese, turkey and chicken.
[2] KC699549, influenza A virus (A/chicken/Egypt/VRLCU-R33/2012(H9N2)).
[3] KC699547, influenza A virus (A/duck/Egypt/VRLCU-R11/2012(H5N1), "backyard duck".
[4] Influenza A virus (A/duck/Egypt/VRLCU-R28/2012(H5N1)), "backyard duck".

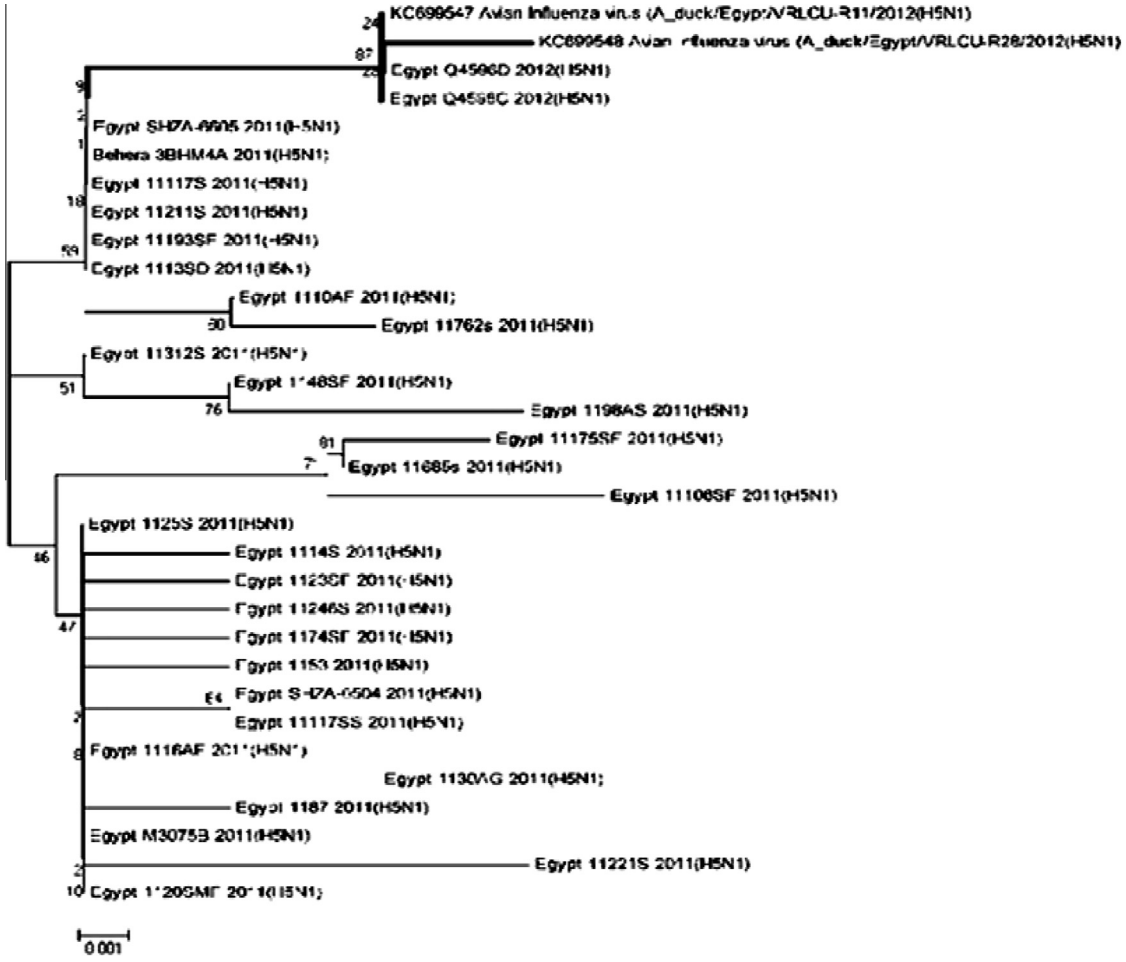


**Figure 1** Genetic characterization of Egyptian H5N1 isolates [analysis of the haemagglutinin (HA1) phylogenetic tree] identified that the viruses are fall within the clade 2.2.1.1. The majority of H5N1 HPAI viruses isolated between 2011 and 2012 are belonging to clade 2.2.1.1., while viruses isolated in 2010 belong to both sub-clades: 2.2.1 and 2.2.1.1.

### *3.1. Antigenic characterization of Egyptian H5N1 viruses*

Antigenic cartography demonstrated for the isolated Egyptian H5N1 HPAI viruses can be intuitively the major antigenic clusters EG-antigen-B, which corresponded to 2.2.1.1 (Fig. 1).

### *3.2. Antigenic characterization of Egyptian H9N2 virus*

Antigenic cartography demonstrated for the isolated Egyptian H9N2 AI viruses can be intuitively grouped-B (Fig. 2). The differences in amino acids between our isolates and previous closely isolates (clade 2.2.1.1) H5N1 can be

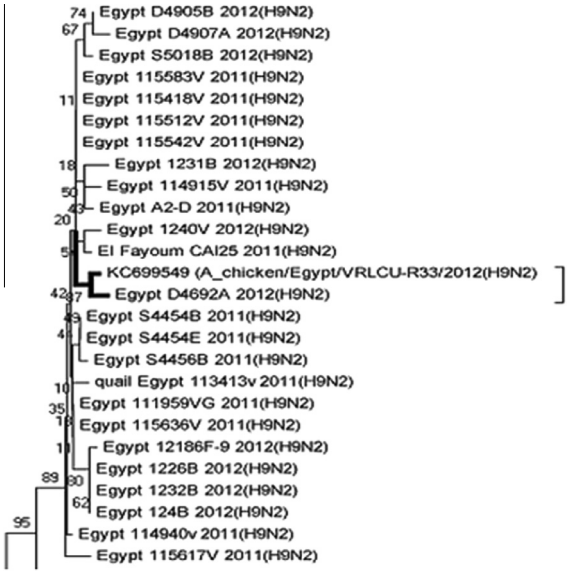


**Figure 2** Genetic characterization of Egyptian H9N2 isolates up to 2012. The figure shows phylogenetic characters of the HA gene of isolated virus in our study KC699549 [A/chicken/Egypt/VRLCU-R33/2012(H9N2)] influenza A virus from the GenBank.

seen in (Fig. 3) and (Fig. 4). Amino acid-changes in the HA molecule of H9N2 isolated in our study are demonstrated in Fig. 4.

## 4. Discussion

### *4.1. H5N1 influenza isolates in Egypt 2012*

Phylogenetic analysis of the HA1 of the two isolated HPAI H5N1 strains {KC699547 [Influenza A virus (A/duck/Egypt/VRLCU-R11/2012(H5N1)] and KC699548 [Influenza A virus (A/duck/Egypt/VRLCU-R28/2012(H5N1)]} showed that, the two isolated HPAI H5N1 strains were genetically very proximate to each other. The differences in amino acids between our isolates and previous closely isolates (clade 2.2.1.1) can be found in residues; 70,103, 110, 119, 25, 102 and 130 in "KC699547" and residues; 13, 58, 61, 91, 99 and 111 in "KC699548". The differences in amino acids (aa) between isolates of 2011 and 2012 were found in residues, 151, 154, 16, 120, 129, 141, 226, 97, 74 and 144.

Antigenic cartography for the first time could demonstrate that, Egyptian H5N1 HPAI viruses intuitively disunited into two major antigenic clusters EG-antigen-A and EG-antigen-

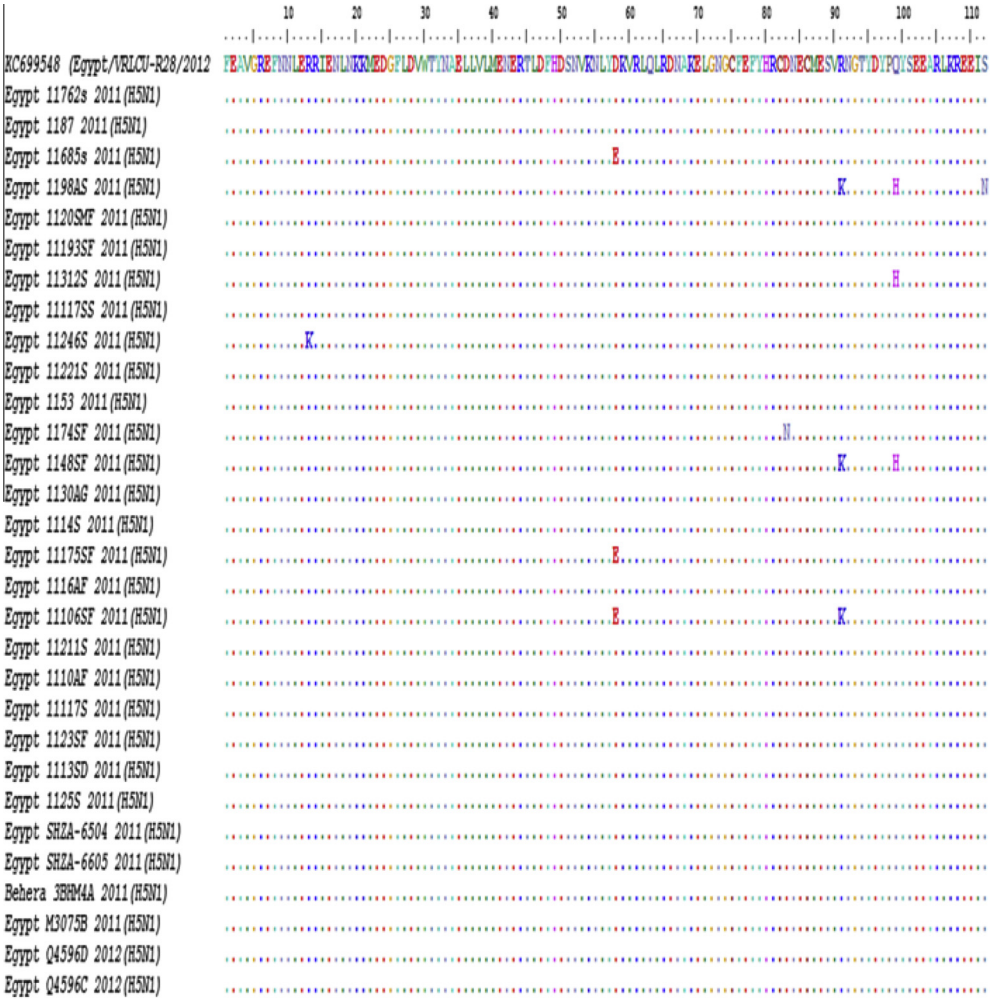


**Figure 3** Amino acid-changes in the HA molecule of H5N1 HPAI isolated in our study KC699547, Influenza A virus (A/duck/Egypt/VRLCU-R11/2012(H5N1).

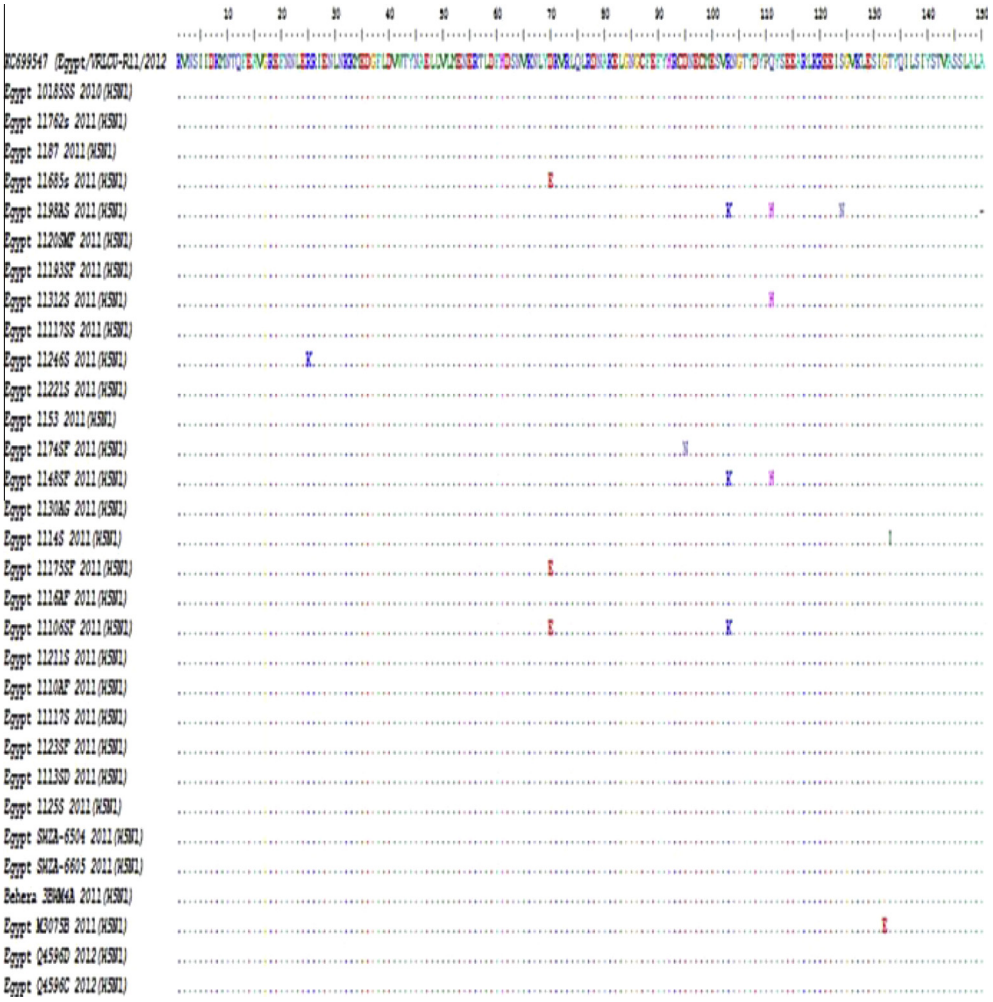

**Figure 4** Amino acid-changes in the HA molecule of H5N1 HPAI isolated in our study KC699548, Influenza A virus ((A/duck/Egypt/VRLCU-R28/2012(H5N1)).

B, which corresponded to clade 2.2.1 and 2.2.1.1, respectively [21]. WHO/OIE/FAO [22] reported that, the H5N1 HPAI Egyptian viruses isolated in 2007 and 2008 were belonging to clade 2.2.1 and clade 2.2.1.1, respectively while, viruses isolated in 2010 were belonging to both sub-clades 2.2.1 and 2.2.1.1.

On 2012 we isolated two strains of H5N1 HPAI which are belonging to genetic clade 2.2.1.1, composed cluster EG-antigen-A. On the other hand, the majority of H5N1 HPAI viruses isolated between 2007 and 2010 were fallen within 2 main genetic groups, already identified by [23] on a smaller data set, and identified as A and B subclades [8]. Three waves of AI spread have occurred in china and Asia proved that H5N1 viruses are unique in having evolved into multiple clades and subclades by reassortment with other influenza viruses in the epicenter of southern China, and accumulation of point mutations [24].

Our findings indicate that, the perpetuating evolution of these H5N1 viruses and the possible establishment of secondary epicentres in Egypt presents a sustained threat to poultry and people globally.

Our results confirm that, Egyptian H5N1 HPAI virus from 2008 exhibited a low cross reactivity in haemagglutination-inhibition (HI) tests against the Mexican vaccine seed strain (H5N2), commonly used in Egypt, suggesting that significant antigenic drift occurred, consequently, our results confirm those reported by Refs. [25,21].

Kim et al. [26] compared the hemagglutination inhibition (HI) titers of sequential isolates, using clade 2.2.1 H5N1 ferret antisera and reported that the HI titers of the viruses isolated from Egyptian domestic poultry in 2007 and 2008 were differed from those of the first viruses isolated in 2006 by a factor of 4 and this indicates only minor antigenic drift.

Since early 2006, H5N1 HPAI has continued to spread in the domestic poultry farms of Egypt despite the implementation of quarantine, amended biosecurity, and vaccination. Although circumstantial evidence fortifies the conception that vaccine pressure is driving this phenomenon, other possibilities for expounding the antigenic drift cannot be ruled out [27]. Whatever the cause, the antigenic drift of H5N1 Egyptian viruses away from the vaccine strain is of concern for the efficacy of vaccination strategies within the poultry industry [28]. The virtually total failure of the vaccine strains to avert viral shedding when birds were challenged with recent field isolates was expected because of the extreme difference in cross-HI and VN activities and greatly reduces the value of

**Figure 5** Amino acid-changes in the HA molecule of H9N2 isolated in our study: KC699549, Influenza A virus (A/chicken/Egypt/VRLCU-R33/2012(H9N2)).

vaccination as a component of a control and eradication program [29].

### *4.2. H9N2 influenza isolates in Egypt 2012*

In the current study, we described the isolation of H9N2 from one sample out of 50 samples of chicken commercial farms was positive for the isolation of one subtype of H9N2; from Sharkeia province [KC699549 (Egypt/VRLCU-R33/2012(H9N2), Group B] as shown in Table 1.

Blast analysis of the nucleotide sequences from the eight viral genes showed that the recently isolated [(Egypt/VRLCU-R33/2012(H9N2)], was proximately cognate to the other Middle East H9N2 strains (Fig. 2). The virus shared the common ancestor A/Qa/HK/G1/97 isolate, which has contributed the internal genes of the H5N1 virus circulating in Asia.

The difference in aa between our isolates and previous closely isolates can be found in residues 33, 47, 65, 90, 92, 143 and 150 in KC 699549 [Egypt/VRLCU-R33/2012(H9N2)] (Fig. 4). H9N2 subtype virus is an eminent member of influenza A genus as it can infect not only avian species but also, although sporadically, mammals such as pigs and humans. The identification of this subtype in multiple host species combined with its co-circulation with other type A influenza viruses may provide a great opportunity to become more virulent through surreptitious spread, mutation and/or reassortment with other subtypes of influenza viruses.

H9N2 virus population circulating in the countries bordering Egypt may be considered the main source for the viruses detected in Egypt; it can be distinguished from the Israeli isolates by a total of 50 key amino acid differences found in the eleven viral proteins with the HA and PB2 genes possessing the majority of these substitutions. The number of mutations and the long branch observed in the phylogenetic tree may suggest a rather long evolution period during which these strains have been circulating undetected in the poultry population.

## 5. Conclusion

Egyptian H5N1-AIVs are perpetually undergoing genetic changes and reveal an involute pattern of drifts. Further experiments are required to attest the role of each residue in affecting antigenic properties for these Egyptians H5N1 viruses. These findings raise the concerns about the value of using influenza vaccines in correlation with the development of antigenic drift in influenza epidemics. H9N2 virus population circulating in the countries bordering Egypt may be considered the main source for the viruses detected in Egypt.

Vaccination, however, cannot be used alone for the control of AI and must be accompanied by other control measures, including quarantines, controlled depopulation and increased surveillance.

## 6. Competing interests

The authors declare that they have no competing interests.

## 7. Authors' contributions

H.K. designed the study, analysed data, H.K. and El-Dahshan wrote the manuscript, H. Kaliefa collected the samples and H.H. and M.R. carried out PCR protocol. All authors read and approved the final manuscript.

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