Research Article

Evolutionary Analysis and Phylodynamics of Avian Influenza Virus H5N1 between 2015 and 2016 in Egypt

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Abstract | Avian influenza viruses (AIVs) continue to pose global threats mainly due to their rapid evolving nature and the dynamic range of susceptible hosts. Zoonotic highly pathogenic avian influenza virus (HPAIV) H5N1 of clade 2.2.1 has recently diversified into two distinct genetic subclades (2.2.1.1 and 2.2.1.2) in Egypt. This study was conducted as part of routine surveillance activities in Egypt; 5202 cloacal and oropharyngeal swabs were collected from live/dead birds, including commercial and backyard flocks, in five different locations in Egypt between the years of 2015 and 2016. All samples were screened using virus isolation, as well as antigenic and molecular detection methods. Full-length hemagglutinin (HA) sequences of six representative H5N1 isolates were analysed to study their genetic evolution followed by estimation of their evolutionary rates among different virus clusters. This analysis revealed a high evolution rate for clade 2.2.1.2. Additionally, analysis of selection pressures in the HA gene revealed a positive selection pressure. Deduced amino acid analysis revealed characteristic mutations at HA antigenic sites besides two other mutations (129Δ, I151T) that were found to be stable in recent subclade 2.2.1.1 isolates from humans and chickens. Our results revealed that linking the epidemiological and sequence data is important to understand the prevalence, transmission, persistence and evolution of the virus, and to monitor the circulating AIV strains and emergence of new AIV subtypes in Egypt. The wide circulation of the 2.2.1.2 subclade, carrying triple mutations (120, 129Δ, I151T) associated with increased binding affinity to human receptors, is an alarming finding with public health importance.

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Introduction

A clade 2.2 avian influenza virus (AIV) was detected in wild birds in Egypt in 2005 and was later isolated from poultry in February 2006, causing severe economic losses to the Egyptian poultry industry (Saad et al., 2007). A clade-based classification system has been established considering the clustering pattern of the HA genes and a total of 10 distinct H5N1 clades (clades 0–9) is now well established (WHO/OIE/FAO H5N1 Evolution Working Group, 2009). These clades can be further classified into sub-clades

due to the continuous evolution of these viruses.

In 2008, H5N1 viruses in Egypt were classified as subclade 2.2.1 and declared to be enzootic, causing severe economic losses to the commercial poultry sector as well as posing a threat to public health due to the zoonotic propensity of the virus (Abdelwhab and Hafez, 2011). By 2012, the WHO/OIE/FAO H5N1 Evolution Working Group recommended that the Egyptian clade 2.2.1 be split into two new subclades, 2.2.1.1; and 2.2.1.2, indicating further divergence of H5N1 viruses circulating in Egyptian poultry (WHO/OIE/FAO H5N1 Evolution Working Group, 2014). From 2007 to 2014, viruses of subclade 2.2.1.1 are being circulated in vaccinated poultry, including turkeys despite intensive vaccination using over 20 diverse H5 vaccines (Abdelwhab et al., 2016). Meanwhile, subclade 2.2.1.2 viruses were observed initially in backyard birds and humans, and recently in commercial birds. The 2.2.1.2 viruses caused severe socioeconomic losses in the poultry industry and posed a serious pandemic threat because of their affinity to human-type receptors (Arafa et al., 2015). Human infections with viruses of the newly emerged 2.2.1.2 subclade amounted to 136 cases with a case fatality rate of 34.8 % in 2015. Therefore, the H5N1 AI situation in Egypt is alarming (WHO, 2016).

The causes of the recent upsurge in poultry outbreaks due to 2.2.1.2 H5N1 infection in parallel of human cases in Egypt remained unclear. There is now ample evidence that the human cases were caused by the newly emerged phylotype 2.2.1.2, for which there was a large increase in the incidence of outbreaks in poultry holdings (Arafa et al., 2016). Thus, there is an urgent need to study and trace the genetic variations among H5N1 viruses circulating in Egypt. In this study, full length HA sequencing and phylogenetic analyses of six HPAI H5N1 viruses isolated during 2015–2016 through systematic surveillance in Egypt were conducted.

Material and Methods

Cloacal and oropharyngeal swabs were collected from five Egyptian governorates during routine AI surveillance between 2015 and 2016, from commercial and backyards sectors; 1187 samples from commercial sectors and 813 samples from backyard sectors during 2015, while in 2016, 2484 samples from commercial sectors and 718 samples from backyard sectors. Pooled swabs were placed in transport medium, consisting of phosphate-buffered saline containing glycerol, penicillin (2000 U/ml), gentamicin (250 mg/ml) and nystatin (500 U/ml). Collected swabs were sent to the National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Egypt for routine diagnosis and further virus characterization. Virus isolation from swab samples was done in 9-day old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) according to OIE guidelines (OIE, 2013). Inoculated eggs were examined daily for 3 – 5 days. Eggs with dead embryos were kept at 4°C for 24 h. Allantoic fluid was collected and examined using the standard hemagglutination test and 1% chicken erythrocytes (OIE, 2013).

Viral RNA was extracted from allanotoic fluid using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RT-qPCR specific for the AIV M gene was performed followed by RT-qPCRs specific for the HA of H5 HA-subtype RT-qPCR (Hoffmann et al., 2007). Complementary cDNA was generated from 4 µl RNA using the OmniScript RT Kit (Qiagen, Hilden, Germany) along with a primer specific to the conserved 12 nucleotides of 3'end of the viral RNA as previously published (Hoffmann et al., 2001). The HA gene segment was amplified using universal primers and 1 µl of cDNA as described before (Kreibich et al., 2009). All PCR reactions were performed in a thermocycler (Eppendorf, Hamburg, Germany) as previously reported (Kreibich et al., 2009). An initial denaturation step (98°C/30 s), followed by 35 cycles each consisting of 98°C/10s, 60°C/30s, 72°C 6 min and final elongation (72°C/5 min) utilizing 2 U Phusion High-Fidelity DNA Polymerase (New England BioLabs, Germany) according to the manufacturer's guidelines. Fragments' sizes were determined by electrophoresis in 1% agar gel in comparison to the DNA 1 kb ladder (GeneRuler™, Thermo Scientific, Germany). Amplicons were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Langen, Germany) Sanger sequencing was conducted in a 3130 Genetic Analyzer (Applied Biosystems). Sequences of full length HA genes from viruses isolated in this study were submitted to the Global Initiative on Sharing All Influenza Data (GISAID) and assigned accession numbers; MF417619 to MF417624. The NetNGlyc 1.0 Server was used to

predict the potential N-glycosylation sites in the surface glycoprotein HA of isolated strains (http://www.cbs.dtu.dk/services/NetNGlyc/).

For the estimation of the rates of nucleotide substitution among H5N1 viruses from Egypt, the Bayesian Markov Chain Monte Carlo (BMCMC) method (BEAST v1.4.7) was applied (Drummond and Rambaut, 2007). The Bayesian GMRF skyride coalescent tree model was used (Minin et al., 2008). The uncorrelated lognormal relaxed (UCLD) clock (Drummond et al., 2006) that allows evolutionary rates to vary along branches within lognormal distributions was used and Hasegawa-Kishino-Yano (HKY) substitution model with empirical base frequencies and gamma site heterogeneity model at 4 categories. Mean evolutionary rates and divergence times were calculated using Tracer V.1.5 (Rambaut and Drummond, 2007).

Sequence similarity to the HA gene of the viruses isolated in this study was probed by Basic Local Alignment Search Tool (BLAST) database available at the NCBI (Altschul et al., 1990). Sequences with the maximum BLAST scores and identity percentages were selected. Moreover, representative Egyptian viruses from subclades 2.2.1.2 and 2.2.1.1 were included in the analysis. All sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh et al., 2014) and further viewed and edited by BioEdit 7.1.7 (Hall, 1990). Amino acid sequences were deduced from gene sequences and identity matrices were calculated using BioEdit. Bayesian inference phylogenetic trees for HA gene segment were generated using MrBayes 3.2.6 (Ronquist et al., 2012) under best-fit models calculated by jModelTest (Santorum et al., 2014). Two parallel runs consisted of four chains of Markov Chain Monte Carlo (MCMC) iterations for 108 generations were selected for each nucleotide sequence. For the phylogenetic tree A/goose/Guangdong/1/1996 (H5N1) (Clade 0) was specified as the out-group. Graphic outputs were produced by FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.91 (www.inkscape.org).

To determine the selection pressure for HA gene segment, 93 sequences of H5N1 viruses circulating in Egypt during 2006–2016 were analyzed by estimating the ratio of non-synonymous (dN) to synonymous (dS) substitutions (ω=dN/dS) across the lineages on a codon-by-codon basis. Selective pressure was defined as follows; ω =1 indicates neutral evolution, ω <1 indicates negative or purifying selective pressure and ω >1 indicates positive selection. The mean values of ω were calculated by the SLAC and FEL methods using the Datamonkey website (http://www.datamonkey.org) (Delport et al., 2010).

Results and Discussion

Although most of the countries earlier infected with HPAI H5N1 viruses have succeeded in eliminating their territories after the initial outbreaks, the virus persists in some South-East Asian countries, including China, Indonesia, Vietnam, and Bangladesh as well as Egypt (FAO, 2013). In spite of previous studies that described the temporal and spatial patterns of HPAI outbreaks that occurred in backyard and commercial poultry sectors in Egypt, there are still obstacles that hinder the improvement of control strategies. First, the mechanism of HPAI H5N1 virus spread and persistence and the role of human-animal interactions with human infections are only partially explored. Secondly, detailed HPAI epidemiological information and data integrity are lacking in the Egyptian poultry industry (Abdelwhab and Hafez, 2011; Arafa et al., 2016).

In the present study, five Egyptian governorates were chosen (Minya, Aswan, Daqahlya, Gharbia and Cairo) in which to study the epidemiological pattern of AI based upon; 1) their locations in Upper and Lower Egypt, 2) high density of rearing and 3) rate of human risk infection with AI. Based on previous reports, H5N1 outbreaks were detected in both Upper and Lower Egypt and affected 96.3% (26/27) of all governorates in Egypt, with higher prevalence in Lower Egypt (Sharkia, Qalyobia, Menufia, Daqahlya and Gharbia) than Upper Egypt (Giza, Fayoum and Minya) (El-Masry *et al.*, 2015). In this study, 2000 and 3202 samples were collected during routine surveillance for AI epidemiology in 2015 and 2016, respectively. During 2015, 1187 samples were collected from commercial sectors, and only 10 cases were positive, while in the same year backyard sectors showed more positive cases with 69 positive cases out of the 813 collected samples. In 2016, 3202 samples were collected (2484 from commercial sectors with only 4 positive cases, and 718 cases from backyard sectors with 31 positive case) (Figure 1). Testing of the collected samples by real time RT-qPCR revealed pos-

itives for type A avian influenza virus with Ct value ranged between 20 and 24. Positive samples were inoculated into ECEs and tested by HA assays, which ranged between 6-9 log^2 from which six representative isolates were chosen for further genetic analysis (Table 1).

To date, Egypt is among the countries with the highest number of human fatalities due to zoonotic H5N1 infections directly related to intense and persistent infection in poultry flocks. Previous studies have suggested that recurrent human infections may be occurring primarily through droplet transmission arising from close contact with infected poultry (Murray and Morse, 2011). In Egypt, the highest incidence remains in the Nile Delta region, where high densities of poultry populations and activities have been established alongside dense human populations. The unorganized marketing networks and poultry's spatial distributions within the human household are possible risk factors for human infections (WHO, 2015).

Influenza H5N1 viruses have five antigenic sites within the HA viral surface glycoprotein, which is a major determinant of influenza virus host range; mutations in these HA sites play an important role in the evolution of H5N1 (Shih et al., 2007). Analysis of antigenic site B (D154, A156 and A184) showed that

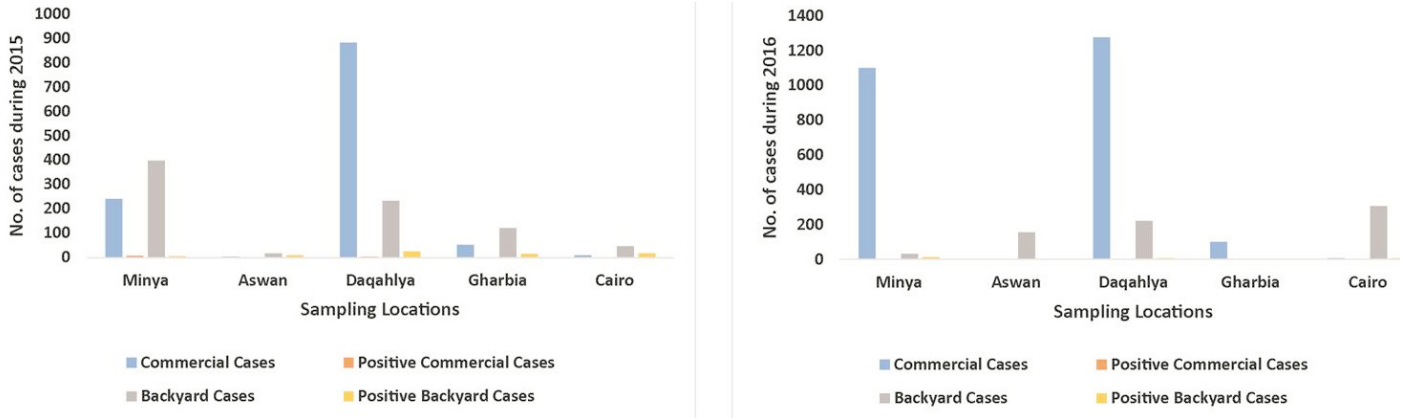

Figure 1: *Geographical distribution for positive cases of highly pathogenic avian influenza H5N1 outbreaks in commercial and backyard flocks within six Egyptian governorates between 2015 and 2016 in compare to the total screened samples.*

Table 1: *Data of the reported isolates.*

Isolate ID	Date	Place*	Breed/Rearing
A/chicken/Egypt/25/2015	12 Feb 2015	Minya	Commercial broiler
A/CK/Egypt/CA110/2015	10 Apr 2015	Cairo	Backyard broiler
A/Egypt/RS57/2015	8 Dec 2015	Gharbia	Backyard broiler
A/chicken/Egypt/CAG139/2016	1 Nov 2016	Daqahlya	Backyard broiler
A/chicken/Egypt/CAL9/2016	15 Jan 2016	Aswan	Commercial broiler
A/chicken/Egypt/S52/2016	23 Jan 2016	Minya	Commercial broiler

Province/Government where specimens were collected from.

Table 2: *Amino acid substitutions at different sites of HA protein.*

Sample ID	Amino acid positions*
A/chicken/Egypt/25/2015	I121F, G245E, Y271H, R473K
A/CK/Egypt/CA110/2015	M226I, A238S
A/Egypt/RS57/2015	K266R
A/chicken/Egypt/CAL9/2016	I117F, D183N, Q187E, R189K, T195N, V210I, K304T
A/chicken/Egypt/S52/2016	I98K, R189K, T195N
A/chicken/Egypt/CAG139/2016	A127T

Amino acid positions according to H5 numbering (Duvvuri et al., 2009).

most of the subclade 2.2.1.2 viruses had mutations D154N. Additionally, seven new sites were described recently as antibody-binding sites (N94, S120, R162, E227, N252, T263 and I282) (Cai et al., 2012). Site E227 was conserved in all Egyptian viruses, whereas subclade 2.2.1.2 viruses possessed mutations S120D, R162K, Y252N and G282S in comparison to the original clade 2.2.1. Moreover, there is another mutation is located in the stalk domain at the oligomerization interface of the HA protein at residue K373R, which is characteristic for the emerging of new clades (Duvvuri et al., 2009) and noted in our isolates in this study. Antigenic drift, the accumulation of point mutations in the viral genome, creates virus variants that escape immunity to previous influenza strains, necessitating continuous alterations to the vaccine strains (Hoelscher et al., 2008). More point mutations were noted at sites along the HA protein, which are provided in Table 2.

After the first introduction of the H5N1 HPAI virus in Egypt in February 2006, a huge number of disease outbreaks was reported in commercial farms because of the newly introduced H5N1 virus, clade 2.2.1 (Aly et al., 2008). The infection was characterized by severe destruction of poultry and widespread occurrence into the country. From December 2007 to May 2008, the emergence of a new variant escape mutant subclade 2.2.1.1 was documented from, primarily, vaccinated flocks. After that, from November 2010 till now; an endemic emerging new subclade 2.2.1.2 was characterized by severe epidemics mainly in backyard poultry as well as commercial sectors and associated with frequent human infections (Arafa *et al.*, 2015). The new subclade 2.2.1.2 showed a high evolution rate over a period of 6 years (6.7×10^{-3}/site/year). Likewise, differences in the nucleotide composition of the coding sequence of HA gene was up to 1.3%, which includes up to fourteen specific fixed nucleotide substitutions distinguishing these viruses from earlier Egyptian isolates before 2014.

Subclade 2.2.1.2 H5N1 viruses have become established in poultry in Egypt, accounting for a majority of human infections in recent years (WHO/OIE/FAO, 2012; Arafa *et al.*, 2016). In Egypt, HA gene of H5N1 are under purifying selection with $0.064<\omega<0.890$, suggesting that a non-synonymous mutation has only 6–89% as much chance as a synonymous mutation of being fixed in the population (Figure 2).

Phylogenetic analysis of these HA sequences was carried out to further identify the genetic spectrum and evolution of H5N1 AIVs which revealed persistent virus evolution and predominance of the endemic subclade 2.2.1.2 cluster in Egypt with establishment of a new cluster for the recent poultry isolates starting from 2015 and human isolates (Figure 3). Subclade 2.2.1.2 viruses has specific signatures (aa D43N, S120D, Δ129S, I151T, R162K, G272S, R325K) which enable efficient replication in human cells (Watanabe *et al.* 2015; Schmier et al., 2015) and several point mutations in the immunogenic epitopes mostly in the HA1 domain were observed which enabled the latter viruses to escape from the humoral immune response induced by H5 vaccines leading to vaccination failure in chickens (Duvvuri et al., 2009; Cattoli et al., 2011). Recent studies demonstrated that five mutations in the H5N1 viruses may be required to enable transmission between ferrets *via* respiratory droplets; these four mutations in HA (Q222L, G224S, T156A and H103Y) and one in the PB2 protein (E627K) (Herfst et al., 2012).

Out of these four mutations at HA protein, there is one mutations (T156A) was identified in all isolates in the present study. Another study identified four HA mutations (N220K, Q222L, N154D and T315I) that improved virus replication and transmission *in vivo* by altering its receptor-binding preference from Sia 2, 3-a-Gal to Sia 2, 6-a-Gal (Imai et al., 2012). Our data showed that only mutation N154D was present in previously circulating Egyptian viruses, but has disappeared from all the strains from the new cluster. Likewise, viruses of the new cluster had several characteristics HA mutations (Table 2). Most of the Egyptian strains possessed three N-linked glycosylation sites at amino acid positions ^{10}N-N-S^{13}, ^{23}N-V-T^{25}and ^{286}N-S-S^{288}. However, viruses from subclade 2.2.1.2 had an additional glycosylation sites at position ^{165}N-N-T^{167} and ^{484}N-G-T^{486} that have been detected in all the isolated viruses in this study when compared with their ancestor H5N1 virus [A/chicken/Egypt/06207-NLQP/2006 (H5N1)].

Progressive evolution of the H5N1 viruses may have occurred in some governorates where multiple clades of viruses have circulated in different years. However, the small number of positive samples from these governorates does not provide strong evidence to support such homogeneity. The fact that outbreaks continue to be reported, despite the widely practised vaccination

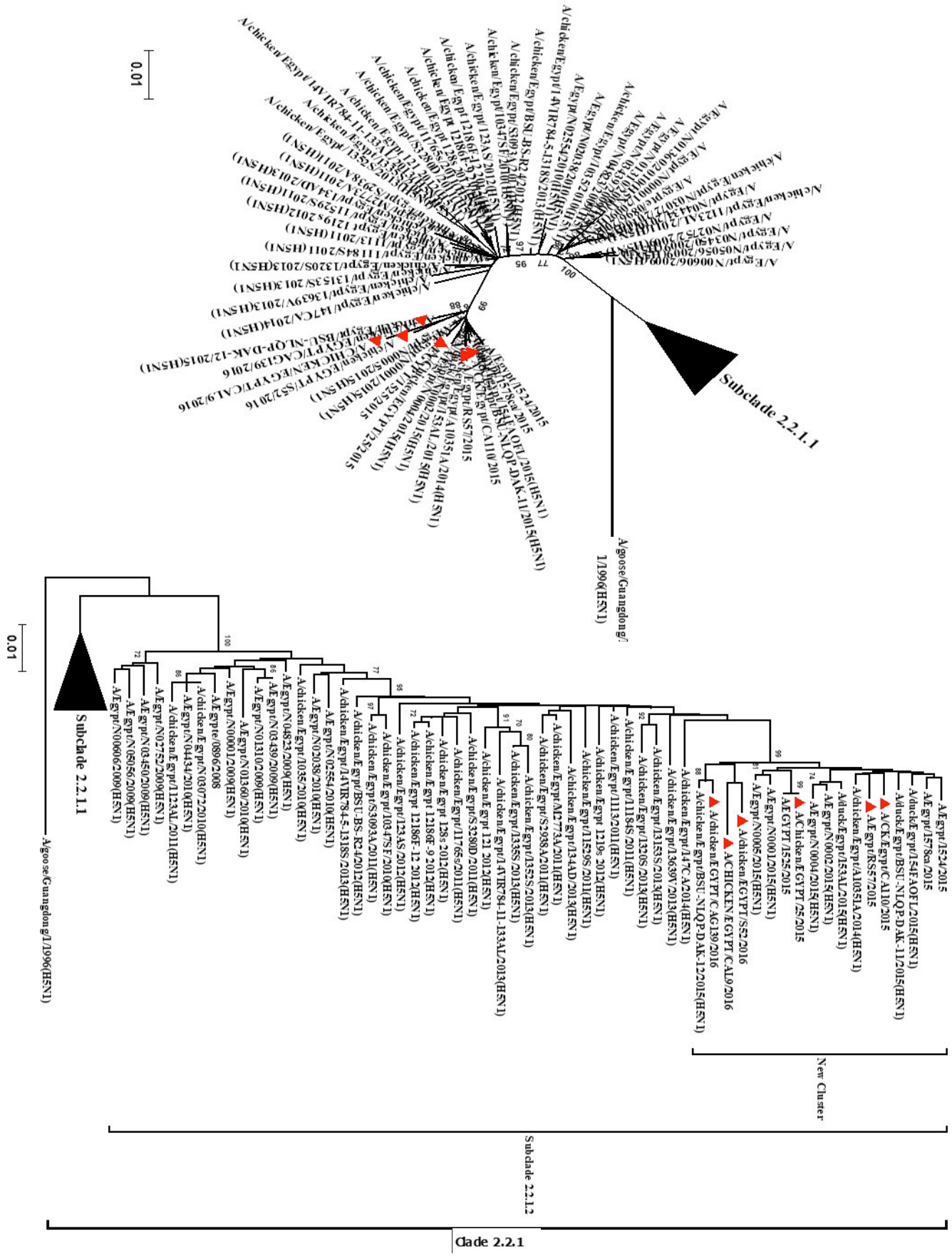

Figure 2: *Phylogenetic tree of haemagglutinin (HA) gene sequences of Egyptian H5N1 viruses showing the continuous evolution of Egyptian H5N1 subclade 2.2.1.2 with the establishment of distinct cluster with close relation to the reported human isolates. The robustness of individual nodes of the tree was assessed using 1000 replications of bootstrap re-sampling of the originally aligned nucleotide sequences. Scale bar represents the number of substitutions per site. The year of isolation and geographical origin of the virus sequences are included in the tree.*

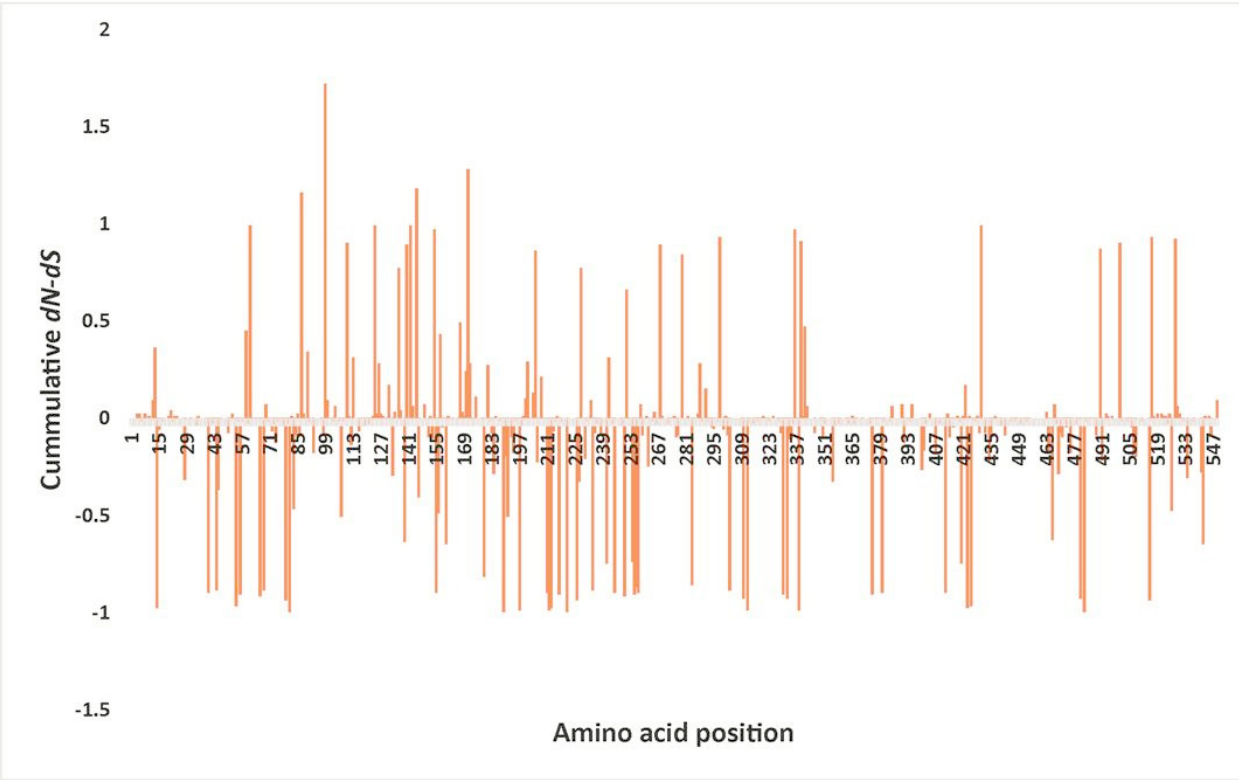

Figure 3: *Distribution of negative and positive selection motifs along the HA protein for the Egyptian H5N1 viruses. Accumulated dN-dS are shown that are spanning amino acid residues from 1 to 547.*

against HPAI H5N1 virus, casts doubt on the effectiveness of the practise in Egypt. Inadequate vaccination coverage and improper application may have contributed to the observed vaccination ineffectiveness. Strategically, quality-assured vaccines should be selected and used in high-risk areas against circulating strains and with consideration for the ease of applications as previous reports described that poor-quality vaccines have led to disease outbreaks (Peyre et al., 2009; FAO, 2011).

In conclusion, a new H5N1 cluster carries signatures from both chicken and human-adapted subclade has been identified. While genetic and biological characterizations were carried out in this study, further research is warranted to investigate the transmission potential of these viruses and to prevent the spread of human-adapted strains in Egypt. Additionally, mutations demonstrated here with unknown biological functions require further investigation.

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Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Conceptualization: A.Arafa, H.A.Hussein, I.Reda. Data curation: O.Elshazly, A.Arafa and H.A.Hussein. Formal analysis: H.A.Hussein and M.A.Rohaim. Investigation: O.Elshazly and H.A.Hussein. Methodology: O.Elshazly and A.Arafa. Software: M.A.Rohaim. Supervision: A.Arafa, H.A.Hussein and I.Reda. Validation A.Arafa, H.A.Hussein and I.Reda. Writing–original draft: O.Elshazly and A.Arafa. Writing–review&editing: M.A.Rohaim and H.A.Hussein.

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