

Emergence of a novel cluster of influenza A(H5N1) virus clade 2.2.1.2 with putative human health impact in Egypt, 2014/15

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A distinct cluster of highly pathogenic avian influenza viruses of subtype A(H5N1) has been found to emerge within clade 2.2.1.2 in poultry in Egypt since summer 2014 and appears to have quickly become predominant. Viruses of this cluster may be associated with increased incidence of human influenza A(H5N1) infections in Egypt over the last months.

In Egypt, highly pathogenic avian influenza (HPAI) influenza A(H5N1) viruses of clade 2.2.1 and their descendants have been circulating in poultry populations since 2006, causing sporadic human infections [1]. Human influenza A(H5N1) infections in Egypt have been reported since the introduction of the virus in 2006 with 204 cases occurring until end of 2014 and a fatality rate of 35,8% in laboratory-confirmed cases reported to the World Health Organization (WHO). However, since January 2015, the incidence of human H5N1 cases in Egypt has increased dramatically: as of 21 March 2015, 116 human cases including 36 deaths have been reported to WHO [2]. This study was initiated to analyse molecular properties of H5N1 viruses that have caused outbreaks in poultry in Egypt since summer 2014 and to compare them with published sequences from H5N1 viruses obtained from recent human cases.

Sample origin

Between October 2014 and February 2015, a new wave of 435 outbreaks of H5N1 infections in poultry in Egypt was reported to the National Laboratory for Quality Control on Poultry Production (NLQP) by Egyptian veterinary authorities (Figure 1). Affected poultry species included chickens, ducks, turkeys and quails on commercial farms as well as in backyard holdings. In this study, 29 H5N1-positive samples, mostly obtained by

passive surveillance and submitted to NLQP for routine analysis, were selected so as to represent different poultry species, sectors of poultry holdings (commercial farms, backyards and live bird markets) and locations (Table 1).

Phylogenetic analyses

Nucleotide sequence data for the haemagglutinin (HA) gene of all 29 viruses and of the neuraminidase (NA) gene of 15 viruses were generated by Sanger sequencing; whole genome sequencing was carried out for four virus isolates selected to represent different locations, moments in time and sectors of poultry holdings.

Phylogenetic analysis of the HA and NA gene sequences was done with the maximum likelihood methodology using the IQTree software [3,4]. The authors gratefully acknowledge the originating and submitting laboratories who contributed sequences used in the phylogenetic analysis to the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database, and recognise in particular Alice Fusaro and colleagues (Istituto Zooprofilattico Sperimentale Delle Venezie, Padova, Italy) as well as Mee Poh (Centers for Disease Control and Prevention, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Influenza Division, Atlanta, United States). Phylogenetic analysis placed the H5N1 viruses sequenced in this study into a separate cluster within the previously defined clade 2.2.1.2 (Figure 2A and 2B). While the H5N1 isolate A/duck/Egypt/14VIR784-4-133AD/2013 (KP035030) harboured the closest related ancestral sequences of this cluster, two further poultry viruses sampled in June and July 2014 were placed directly at the root of this cluster: A/quail/Egypt/BSU5514-AR2219/2014 (EPI557138) was at the basis of the HA phylogeny while A/duck/

TABLE 1

Influenza A(H5N1) viruses included in this study and collected from poultry, Egypt, June 2014–January 2015 (n=29)

No	Sample ID	Sequenced	Collection date	Governorate	Source	Accession number (EpiFlu)
1	A/turkey/Egypt/14139FAOS/2014	Whole genome	18 Jun 2014	Assiut	LBM	EPI574374–81
2	A/duck/Egypt/14154FAOS/2014	HA, NA	25 Jun 2014	Monofiya	LBM	EPI573331–2
3	A/chicken/Egypt/1427AF/2014	HA	3 Sep 2014	Fayoum	farm	EPI573333
4	A/chicken/Egypt/1476CA/2014	HA	14 Oct 2014	Al-Gharbiya	Household	EPI573330
5	A/turkey/Egypt/ AR235-S240NLQP/2014	HA, NA	31 Oct 2014	Cairo	Household	EPI573251–2
6	A/turkey/Egypt/14240FAOS/2014	Whole genome	31 Oct 2014	Cairo	LBM	EPI574382–89
7	A/chicken/Egypt/141/2014	HA	3 Nov 2014	Aswan	Household	EPI573334
8	A/duck/Egypt/14227FAOS/2014	HA	4 Nov 2014	Sohag	Household	EPI573335
9	A/chicken/Egypt/ AR234-FAOF8NLQP/2014	HA, NA	12 Nov 2014	Menia	Farm	EPI573249–50
10	A/chicken/Egypt/ AR231-CA113NLQP/2014	HA, NA	19 Nov 2014	Suez	Household	EPI573243–4
11	A/chicken/Egypt/1478CAL/2014	HA	23 Nov 2014	Qena	Household	EPI573318
12	A/duck/Egypt/144/2014	HA	4 Dec 2014	Giza	Household	EPI573323
13	A/chicken/Egypt/14140CA/2014	HA	5 Dec 2014	Assiut	Household	EPI573329
14	A/turkey/Egypt/ AR238-SD177NLQP/2014	Whole genome	6 Dec 2014	El-Beheira	Household	EPI573261–8
15	A/chicken/Egypt/14148CA/2014	HA	9 Dec 2014	Menia	Household	EPI573314
16	A/duck/Egypt-BS/146RS-f6/2014	HA	14 Dec 2014	Beni Suef	Farm	EPI573315
17	A/duck/Egypt/1427SL/2014	HA	14 Dec 2014	Sohag	Household	EPI573316
18	A/chicken/ Egypt/AR233-S283NLQP/2014	HA, NA	15 Dec 2014	Qena	Household	EPI573247–8
19	A/chicken/Egypt/14168CA/2014	HA, NA	16 Dec 2014	Menia	Household	EPI573327–8
20	A/duck/Egypt/ AR232-A13NLQP/2014	HA, NA	22 Dec 2014	Giza	Household	EPI573245–6
21	A/chicken/Egypt/152RS/2015	HA	29 Dec 2014	Monofiya	Household	EPI573322
22	A/chicken/Egypt/152/2015	HA	1 Jan 2015	South Sinai	Household	EPI573319
23	A/chicken/Egypt/153AF/2015	HA, NA	4 Jan 2015	Fayoum	Household	EPI573320–1
24	A/chicken/Egypt/1540S/2015	HA, NA	11 Jan 2015	Assiut	Household	EPI573336–7
25	A/chicken/Egypt/152AI/2015	HA, NA	11 Jan 2015	Ismailia	Household	EPI573325–6
26	A/chicken/Egypt/1510CA/2015	HA, NA	14 Jan 2015	Cairo	Household	EPI573312–3
27	A/duck/Egypt/ AR236-A3NLQP/2015	Whole genome	15 Jan 2015	Giza	Household	EPI573253–60
28	A/duck/Egypt/1560S/2015	HA	18 Jan 2015	Dakahlia	Household	EPI573324
29	A/chicken/Egypt/1575S/2015	HA	21 Jan 2015	Assiut	Household	EPI573317

HA: haemagglutinin; LBM: live bird market; NA: neuraminidase.

Egypt/14154-FAOS/2014 (EPI573331) marked the basis of the NA tree (Figure 2A and 2B, green colour). The cluster has expanded since October 2014; the fact that no more sequences of the older range of 2.2.1.2 viruses were detected thereafter indicates that this cluster had become predominant over previously circulating phylogenotypes. GenBank sequences of two recent H5N1 HPAI viruses obtained from infected humans in Egypt in November 2014 (AJM70734 and AJM70746), fell into the same expanding cluster (Figure 2A and 2B, blue colour). Calculation of the time to the most recent common ancestor (TMRCA) of the emerging phylogroup by BEAST analysis (Figure 2C) [5] suggested that ancestors of this phylogroup emerged around February 2014. Similar phylogenetic relationships were observed for the internal gene segments of these viruses (data not shown, available from authors upon request). The phylogenetic

information of the internal genes supports the results for the HA and NA gene sequences, indicating that the new viruses represent a distinct cluster that originated from previously circulating viruses of clade 2.2.1.2. So far, no reassortment events were found to be involved in generating this newly emerging phylogroup.

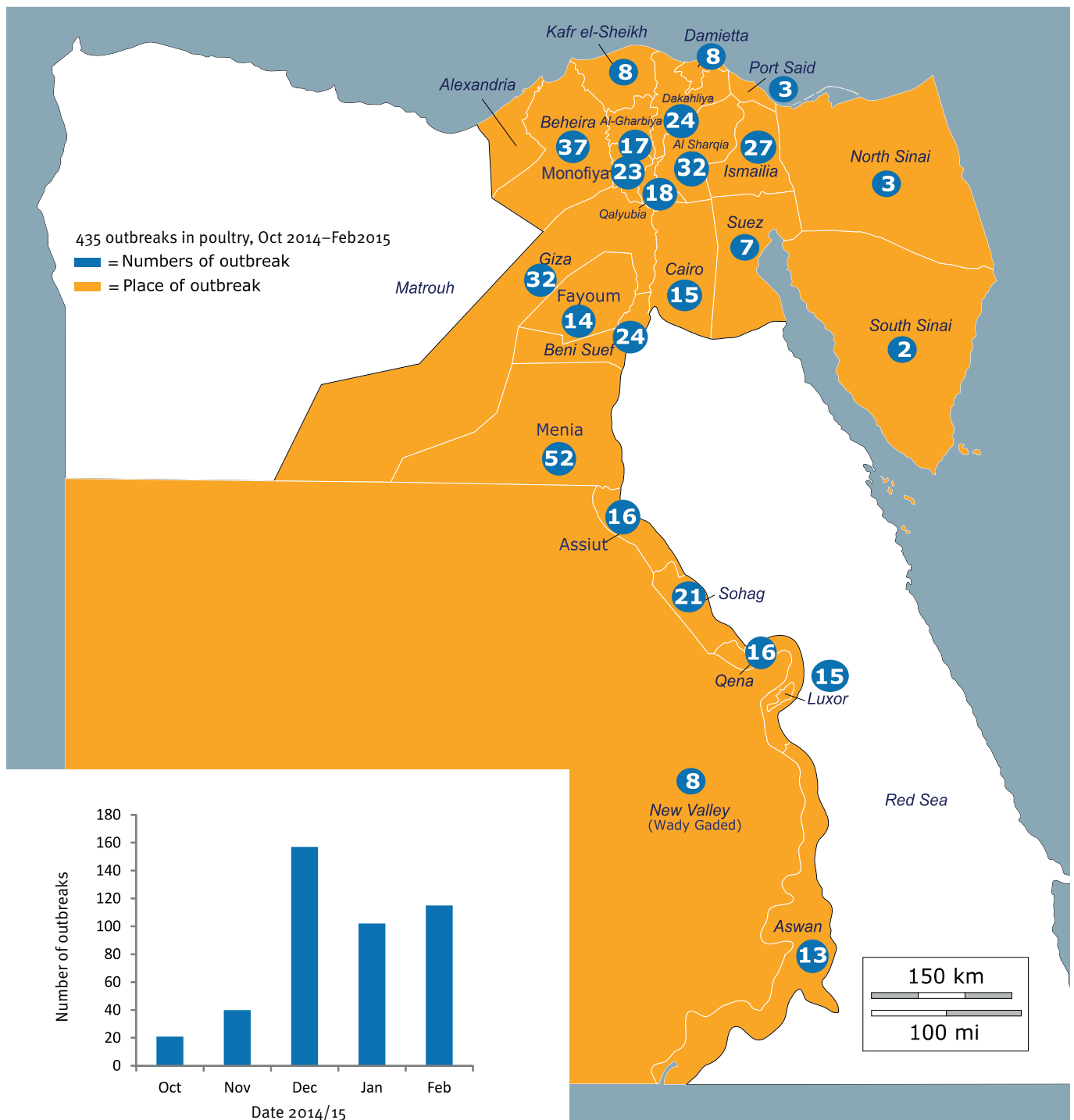
Genetic characterisation

Compared with viruses sampled before October 2014 in Egypt, the emerging cluster contained distinct fixed mutations in several genome segments (PB2, PB1-F2, HA, NA, M1).

In the HA gene, differences in the nucleotide composition of the coding sequence of up to 1.3% were found, which included up to 14 specific fixed nucleotide substitutions distinguishing these viruses from earlier

FIGURE 1

Temporal (graph) and geographic (map) distribution of outbreaks of highly pathogenic avian influenza A(H5N1) in poultry, Egypt, October 2014–February 2015 (n=435)



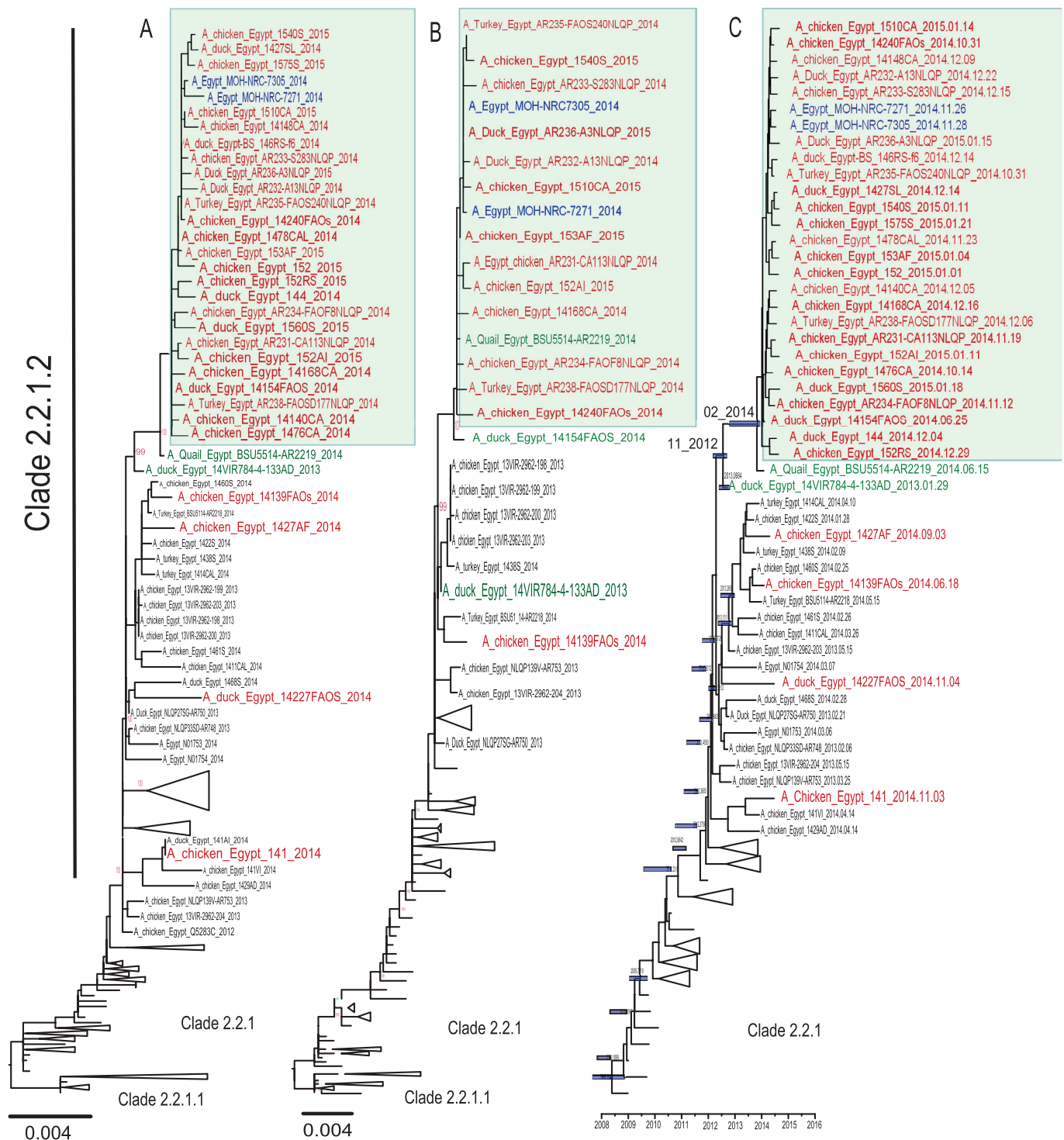
Source of the map: d-maps.com.

Egyptian isolates of 2014. A total of 12 of these mutations were synonymous (silent) and only two resulted in amino acid substitutions (K373R and F537S). Of these, only K373R, located in the stalk domain at the oligomerisation interface of the HA, is characteristic of the emerging cluster; this mutation has sporadically been reported in very few older isolates from Egypt [6].

The mutation F537S had already been detected in several older isolates. In addition, the HA protein of the viruses in the new cluster contained mutations D94N, T156A, K189R and P235S, which are associated with improved binding to SA α 2,6-Gal, the human type of influenza virus receptors [7]. However, these mutations were present also in earlier clade 2.2.1 H5N1 viruses

FIGURE 2

Phylogenetic analysis of the HA (A) and NA (B) genes (coding regions) and maximum clade credibility tree (C) based on the HA open reading frame of selected highly pathogenic avian influenza A(H5N1) viruses, Egypt, June 2014–January 2015 (n=29)



HA: haemagglutinin; NA: neuraminidase.

The phylogenetic analysis was done by maximum likelihood method using the IQTree algorithm [3,4]. BEAST [5] analysis was carried out to determine time to the most recent common ancestor (TMRCA; indicated by horizontal blue bars overlaying nodes). TMRCA calculations were based on an uncorrelated log-normal relaxed clock model. The maximum clade credibility tree was scaled to time using the collection dates (day/month/year) of all samples. Red labels indicate poultry viruses sampled after August 2014 and sequenced in this study. Blue labels denote sequences of viruses retrieved from human influenza A(H5N1) infections (AJM70734; AJM70746). Green labels highlight viruses close to the basis of the emerging cluster of selected H5N1 HPAI viruses circulating in Egypt.

The authors gratefully acknowledge the originating and submitting laboratories who contributed sequences used in the phylogenetic analysis to the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database.

TABLE 2

Antigenic characterisation of highly pathogenic avian influenza A(H5N1) viruses from Egypt and elsewhere, based on haemagglutination inhibition assays

Influenza antigens for immunisation	Immune sera	H5 LP		H5N1 HP				
		Am	EA	2.2.1	2.2.1N ^a	2.2.1.1	1	2.3.2.1
		A/H5N2 MX	A/H5N2 PTD	R740/09	AR238/15	R737/09	NIBRG-14	R1970/13
A/H5N2 LP, Mexico vaccine	S1	9	8	3	3	<1	7	<1
	S2	9	7	5	5	<1	5	1
	S3	10	8	6	5	<1	6	1
A/H5N2 LP, Potsdam (Germany)	S4	7	8	5	5	<1	7	3
	S5	8	10	8	8	<1	8	5
	S6	7	8	7	6	<1	5	<1
A/H5N1 R65	S13	7	7	8	7	3	7	6
A/H5N1 R737	S7	3	4	5	5	9	5	6
	S8	2	1	5	4	10	4	5
	S9	2	<1	3	3	10	3	4
Re-5	S10	6	6	3	3	<1	6	4
	S11	5	6	4	4	2	6	4
	S12	4	5	4	4	4	6	4
None	S-SPF	<1	<1	<1	<1	<1	<1	<1

Am: American; EA: Eurasian; HP: highly pathogenic; LP: low pathogenic; PTD: Potsdam.

^a 2.2.1N denotes a virus representative of the currently emerging cluster of highly pathogenic avian influenza A(H5N1) viruses in Egypt.

Immune sera were raised in chickens against low pathogenic (LP) A/chicken/Mexico/232/1994 (A/H5N2 LP, used as a vaccine virus and representing an American LP H5 strain), against A/duck/Potsdam/1402-6/1986 (A/H5N2 LP PTD, representing an Eurasian LP H5 strain) and against highly pathogenic (HP) A/whooper swan/Germany/R65/2006 (A/H5N1 R65, clade 2.2), A/chicken Egypt/0879-NLQP/2008 (A/H5N1 R737, clade 2.2.1.1) and reverse genetically-modified (rg) A/duck/Anhui/0.5006 (Re-5, clade 2.3.4). A specific pathogen-free chicken serum served as negative control. All viruses used for immunisation, except Re-5, also served as antigens in HI assays. Additional antigens were derived from A/chicken/Egypt/083-NLQP/2008 (R740/09, clade 2.2.1), A/turkey/Egypt/FAO-SD177/2015 (AR238/15, clade 2.2.1, emerging cluster), rg A/Vietnam/1194/2004 (NIBRG-14; clade 1) and A/Hill myna/Austria/R1970/2013 (R1970/13, clade 2.3.2.1). Homologous (same clade) serum-antigen reactions are shown in bold.

in Egypt. Since no substituting mutations were found in HA epitopes, we do not expect marked differences in the antigenic properties of the emerging phylotype compared with the previously circulating clade. This was partially confirmed by haemagglutination inhibition assays using sera against different clades of H5 viruses (Table 2).

The NA gene of the emerging cluster differed by seven nucleotide substitutions from recent H5N1 HPAI viruses of clade 2.2.1.2. Four of the seven mutations encoded amino acid substitutions not previously reported in 2.2.1.2 viruses: V34I, I74V, V244I and V284I (Table 3). Mutation V34I has been reported in H5N1 strains from Cambodia from 2013 where an increase in human H5N1 infections was observed [8]. No biological function has been associated with the V34I substitution, while positions 74, 244 and 284 are located in B- or T-cell antigenic regions of the NA protein [9].

For four viruses, whole genome sequences were generated and further signature mutations of the emerging viruses were found in the internal gene segments as well: Non-silent cluster-specific mutations were confined to the PB2 (M66I, T106A), PB1-F2 (G22E), and M1 (I15V) proteins (Table 3).

Discussion

Influenza pandemics remain one of the major threats posed by communicable diseases to the human population. The avian reservoir of influenza viruses contributed by reassortment to the emergence of most previous pandemic human influenza viruses [10]. Since their emergence in Asia in 2003, HPAI viruses of subtype H5N1 and their recent descendants continue to cause significant economic losses to commercial poultry not only in Asia, but also in Egypt where high mortality in poultry has continuously been observed since 2006 [11, 12]. They also exhibit strain-specific zoonotic potential resulting in sporadic avian-to-human spillover transmissions which lead to human infections associated with a high case fatality rate [13]. However, apart from sporadic cases (e.g., family clusters) sustained human-to-human transmission of any of these viruses has not ensued so far.

Our data confirm the emergence of an additional virus cluster within the Egyptian 2.2.1.2 clade of H5N1 HPAI viruses. Since November 2014, viruses of this new cluster appear to have become dominant over the previously described clade 2.2.1.2 phylotypes circulating

TABLE 3

Amino acid residues distinguishing recently emerging highly pathogenic avian influenza A(H5N1) viruses from virus lineages circulating before November 2014, Egypt, March 2015

Gene segment	Position	New cluster	clade 2.2.1.2	clade 2.2.1.1
PB2	66 ^a	I	M	M
	106 ^a	A	T	T
PB1-F2	22	E	G	G
HA	389 ^a	R	K	K
NA	34	I	V	V
	74	V	I	I
	244	I	V	V
	284	I	V	V
M1	15	V	I	I

HA: haemagglutinin; NA: neuraminidase.

Data on the new cluster are based on sequences established in this study: HA (29 sequences), NA (15 sequences), internal gene segments (4 sequences). Data for older clades were retrieved from public databases.

^a Some of the listed mutations have been infrequently observed among single isolates from previous years.

in various poultry species. The only two publicly available sequences of viruses isolated from recent human H5N1 cases in Egypt show similar mutation patterns and fall into the same phylogenetic group. The molecular determinants that may improve the evolutionary fitness of these viruses need to be further clarified. The emergence of new clusters of H5N1 HPAI viruses in Egypt is not without precedence: In late 2007, a subclade of antigenic drift variants, later designated 2.2.1.1, emerged and expanded (clade 2.2.1.1a) in commercial poultry in Egypt but disappeared until end of 2010 [14] and, contrary to the current situation, did not replace 2.2.1 viruses. Viruses of clade 2.2.1.1 that emerged in 2007 hardly caused any human cases: according to the OpenFlu database [15]: only one of 100 H5N1 isolates from humans in Egypt belonged to clade 2.2.1.1; all others belonged to clade 2.2.1 and 2.2.1.2. In contrast, the emerging cluster identified in this study seems to be predominant across all poultry production sectors and has already caused a third of all human infections reported in Egypt since 2006 in only three months of 2015.

Given the endemic status of influenza H5N1 in poultry and the limitations of the reporting system of H5N1 HPAI virus outbreaks in poultry in Egypt, it is difficult to assess whether the altered epidemiological pattern of the emerging phylotype is due to altered biological properties in poultry or whether the increased incidence of infections in poultry merely reflects an increased viral burden across all poultry sectors in Egypt. In any case, the observed recent rise in outbreaks in poultry probably resulted in increased exposure risks for humans in contact with poultry, which may have caused

an increased incidence in human cases. However, it can at this point not be excluded with certainty that the emerging phylotype of viruses may have increased zoonotic potential and may be transmitted more efficiently to humans, although this assumption cannot be drawn from the molecular evidence described here. Further studies of the pathogenicity and transmissibility of these viruses in humans, e.g. in the ferret model, are required. Concerted efforts of both veterinary and public health authorities are urgently needed to interrupt virus circulation in poultry in Egypt efficiently. This will help decrease the risk of human exposure to the virus.

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Conflict of interest

None declared.

Authors' contributions

Abdel-Satar A. Arafa, Mahmoud M. Naguib and Timm Harder conceived the study. Walid H. Kilany, Ahmed Samy and Ahmed Abdelhalim were involved in the collection, identification and isolation of viruses from field samples. Christine Luttermann, Naglaa Hagag and Abdullah A. Selim conducted the Sanger sequencing. Christian Grund provided data from haemagglutination inhibition assays. Mahmoud M. Naguib, Abdel-Satar A. Arafa, E. M. Abdelwhab and Timm Harder produced, analysed and interpreted genetic and phylogenetic data. Gwenaëlle Dauphin, Yilma Makonnen and Mohamed K. Hassan provided and analysed epidemiological data. Timm Harder and Mahmoud M. Naguib drafted the manuscript, Thomas C. Mettenleiter, Martin Beer, Juan Lubroth and all co-authors critically analysed and revised the manuscript and provided final approval.

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