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Molecular Evolution of *Hemagglutinin* (*HA*) Gene of H5N1 Avian Flu Virus Isolated from Chickens, Ducks and Human Cases in Egypt

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ABSTRACT

The molecular phylogenetic tree of the *Hemagglutinin* (*HA*) gene within and between (H5N1) viral isolates from birds and human cases in Egypt was constructed and analyzed by using Laser gene 7.1 software. The *HA* gene data set was downloaded from GenBank in January 2008. Results showed that teal duck H5N1 virus isolates (GenBank accession nos. EF042623 and EF042624) formed the root of the phylogenetic tree of all H5N1 isolates within and between hosts. The human H5N1 isolate (GenBank accession nos. EF535825), which was isolated from El-Menia governorate, was the root of all infected human cases while chicken isolates, which originated in Cairo and Giza governorates, in addition to the WHO strain (GenBank accession nos. DQ837588, DQ837589 and EU146879, respectively) were found to be the roots of the phylogenetic tree of infected chickens. The *HA* gene had varied sequence length (bp) in the different viral isolates ranging between 1704 and 1743 bp. These results suggest that the outbreak of avian flu infection in Egypt, which had first been reported in 2005, was transmitted by migrating birds, especially teal ducks, and that infection was concentrated only in the east side of Egypt extending from Damietta to Aswan rather than the west side of Egypt based on the survey of *HA* gene flat files which were submitted to GenBank. The *H5 HA* genes have high mutation and reassortment rates which evolved rapidly due to their circulation in birds and humans. The authors suggest that the H5N1 isolates of Cairo, Giza and El-Menia (GenBank accession nos. DQ837588, DQ837589, EU183325 and EF535825, respectively) will be good candidates for vaccine production.

Keywords: contiguous diseases, low pathogenic H5N1, molecular phylogenetics, sequence alignment, vaccination strategy, virus virulence

INTRODUCTION

Avian flu viruses are considered to be the most pandemic viruses that infects both birds and mammals (Fan *et al*. 2009), including humans and pigs. In 1918, 50 million people died due to Spanish flu (H1N1). Several decades later, two isolates of influenza pandemic viruses, H2N2 and H3N2, were recorded in 1957 and 1968, respectively (Webby and Webster 2003).

HA codes for hemagglutinin, an antigenic glycoprotein found on the surface of influenza viruses, and is responsible for binding the virus to the cell that is being infected. NA codes for neuraminidase, an antigenic glycosylated enzyme found on the surface of the influenza viruses. It facilitates the release of progeny viruses from infected cells. The HA and NA RNA strands specify the structure of proteins that are most medically relevant as targets for antiviral drugs and antibodies. HA and NA are also used as the basis for naming different subtypes of influenza A viruses. This is the origin of H and N in H5N1 (Couch 1996).

There are different molecular subtypes of the two surface lipoproteins of H5N1, HA (HA1 to HA16) and NA (NA1 to NA9), based on serological and molecular techniques e.g. ELISA and PCR (Fouchier *et al*. 2005). In 1996 and 1997 a highly pathogenic H5N1 (influenza A virus) avian flu isolates emerged in Hong Kong and in October 2007, 53 countries were invaded by the H5N1 isolates causing outbreaks in poultry and wild birds causing the death of 151 people (Wan *et al*. 2007).

Although humans are rarely infected with H5N1 viruses, up to 400 people in different parts of the world, Asia, Africa, Eastern Europe, and the Middle East, have been infected and in 2003 about 63% of those infected died (Pattnaik *et al*. 2006).

Since 2003 in Egypt, the US Naval Medical Research unit No. 3 and the Egyptian Ministry of Environment collaborated in collecting samples from migratory birds to detect circulating influenza viruses.

In February 2006 the Egyptian health authorities reported the outbreak of H5N1 in poultry and humans according to World Health Organization (WHO) records. Vaccination based on neutralizing antibodies requires accurate prediction of the used viral candidate antibody production, especially since these types of antibodies are highly specific to the level of subtype and strain.

Saad *et al.* (2007) reported that a total of 203 cloacal swabs of migratory bird samples of either live bird markets or caged birds trapped by fishermen in the Nile delta region were positive for influenza A virus when tested at the molecular biology level by specific primers for the matrix gene (coding for the matrix proteins M1 and M2) and the *HA* gene (coding for the HA protein) which confirmed the existence of avian influenza virus in Egypt.

The main obstacle for vaccine production is to determine and predict the most suitable and effective candidate isolate and the time required for vaccine production (Epstein 2002).

The authors concentrated their efforts on predicting the evolution of the Egyptian H5N1 viruses isolated from chickens, ducks and humans by assessing the phylogenetic relationship for all Egyptian H5N1 viruses to identify the common ancestor for the *H5* gene, so as to identify the possible source of primary bird infection, especially in infected poultry in Egypt.

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MATERIALS AND METHODS

In this study the nucleotide sequences of *H5* genes from 28 chickens, 9 ducks and 13 human cases were available from GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers shown in **Tables 1-3** during the period from December 2005 to January 2008.

Table 1 Accession numbers, origin of isolation, year of isolation and sequence length of H5 genes isolated from infected chickens in Egypt.

H5 gene sequence length	Year	Origin of isolation	ISDN/NCBI Accession number of the H5N1 virus
1707 bp	2006	Beni-Suef	EU183324
1707 bp	2006	Beni-Suef	EU183327
1707 bp	2006	Gharbiya	EF441279
1741 bp	2006	Gharbiya	EF469651
1707 bp	2006	Fayoum	EU183323
1595 bp (Partial)	2006	Fayoum	DQ837587
1707 bp	2006	Menofia	EF441276
1707 bp	2006	Damietta	EF441278
1604 bp (Partial)	2006	Giza	DQ837589
1741 bp	2006	Sharqiya	EF469652
1613 bp (Partial)	2006	Cairo	DQ837588
1673 bp (partial)	2006	_	DQ447199
1707 bp	2006	_	DQ862001
1596 bp (partial)	2006	_	EF042622
1671 bp (partial)	2006	_	EU146879
1698 bp (partial)	2006	_	EU146866
1700 bp (partial)	2006	_	EU146880
1705 bp	2007	Beni-Suef	EU183328
1704 bp	2007	Beni-Suef	EU183332
1704 bp	2007	Beni-Suef	EU183329
1707 bp	2007	Beni-Suef	EU183326
1707 bp	2007	Beni-Suef	EF441277
1707 bp	2007	Gharbiya	EF441280
1741 bp	2007	Gharbiya	EF469660
1738 bp	2007	Gharbiya	EF469659
1741 bp	2007	Gharbiya	EF469654
1741 bp	2007	Fayoum	EF469650
1741bp	2007	Giza	EF469653

Table 2 Accession numbers, origin of isolation, year of isolation and sequence length of H5 genes isolated from infected ducks in Egypt.

H5 gene sequence length	Year	Origin of isolation	ISDN/NCBI Accession No. of the H5N1 virus
1197 bp (partial)	2005	Damietta	EF042623
1596 bp (partial)	2005	Damietta	EF042624
1707 bp	2006	Menia	EU183325
1741 bp	2006	Gharbiya	EF469655
1715 bp (partial)	2006	Menofia	EF469656
1707 bp	2006	-	DQ862002
1704 bp	2007	Beni-Suef	EU183331
1741 bp	2007	Giza	EF469657
1707 bp	2007	Gharbiya	EF441281

Table 3 Accession numbers, origin of isolation, year of isolation and sequence length of H5 genes isolated from infected humans in Egypt.

H5 gene sequence length	Year	Origin of isolation	ISDN/NCBI Accession No. of the H5N1 virus
1639 bp (partial)	2006	Gharbiya	EF061116
1692 bp (partial)	2006	-	EU146867
1686 bp (partial)	2006	-	EU146868
1730 bp	2007	Aswan	EF535824
1730 bp	2007	Aswan	EF535822
1730 bp	2007	Aswan	EF535823
1730 bp	2007	Menia	EF535825
1713 bp	2007	Fayoum	EF535817
1716 bp	2007	Fayoum	EF535818
1727 bp	2007	Qena	EF535826
1716 bp	2007	Daqahliya	EF535820
1730 bp	2007	Daqahliya	EF535821
1717 bp	2007	Sharqiya	EF535819

Phylogenetic and molecular analysis

The sequences of the *HA* gene within and between infected chickens, ducks and humans were initially aligned with the Megalign program in DNASTAR package software Using Clustal V alignment algorithm and the results were confirmed by Clustal X software.

Also, the phylogenetic tree of *HA* genes was constructed using the neighbor-joining method in Lasergene 7.1 software (DNASTAR. INC, Madison, Wi, USA).

RESULTS AND DISCUSSION

Molecular size analysis of *H5* genes (chickens)

According to the available *HA* (*H5*) gene data set, the molecular (sequence) size of H5 sequences among infected chicken isolates ranged from 1741 to 1707 bp (**Table 1**). There was a difference in the number of infected chickens within different Egyptian governorates while the *H5* genes in the 28 infected chickens had varying sequence lengths.

According to H5 sequences submitted to GenBank, there was a tendency for the molecular size (sequence length) of the *H5* genes to decrease from the north to the south of Egypt: 1741 bp in Gharbiya and Sharqiya isolates but 1705 bp in Beni-Suef isolates. These variations in sequence size could be due to either a continuous forward or back mutation process which occurred in the *HA* gene of H5N1 viruses during its circulation in birds resulting in the evolution of a new sublineage in 2007 (Pattnaik *et al*. 2006). These observations would raise a question: "Does a relationship exist between virulence and nucleotide sequence lengths?"

There is a positive relation between virulence and nucleotide length observed from the increasing number of infected chickens from 2006 to 2007 (**Table 1**).

The evolution of H5N1 viruses in recent years has been associated with increasing numbers of infected birds and expansion of its host range, which include terrestrial poultry, wild birds, pigs and humans (Webster *et al*. 1992; Chen *et al*. 2004; Keawcharoen *et al*. 2004; Kuiken *et al*. 2004).

Phylogenetic analysis of H5 genes (chickens)

The percentage of nucleotide divergence in the *HA* gene sequence between Egyptian virus isolates of infected chickens was calculated using the 'Megalign' program (**Table 4**).

The *H5* genes in Egyptian virus isolates were very similar to each other (99.1-100%), which implies a low level of genetic variation (<1%).

The variation in nucleotide sequence of *H5* genes between Egyptian isolates might have occurred during transmission and virus circulation in chickens.

The phylogenetic relationship between H5N1 viruses which circulated in several regions of Egypt during 2006-2007 (**Fig. 1**) showed three sub-clades: sub-clade 1 includes Beni-Suef isolates (7 isolates), Gharbiya (2 isolates) and one isolate from Menofia, Fayoum and Damietta in addition to 2 isolates from an unknown source. This sub-clade evolved from Beni-Suef and Gharbiya isolates (GenBank accession nos. EF441277 and EF441279), respectively. Sub-clade 2, includes Gharbiya isolates (4 isolates) and one isolate each from Giza, Fayoum and Sharqiya. This sub-clade originated from the Giza isolate (GenBank accession no. EF469653). Sub-clade 3 includes isolates from Cairo, Giza and Fayoum in addition to WHO isolates (4 isolates). This sub-clade originated from the Cairo isolate (GenBank accession no. DQ837588).

The *H5* gene sequences of all chickens have a common ancestor (root) which evolved from the Cairo isolate (GenBank accession no. DQ837588).

Table 4 Nucleotide sequence homology of HA gene among Egyptian Chicken'S H5N1 influenza virus.

		Percent identity																													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		
	1		99.6	99.5	99.5	99.1	99.1	99.1	98.8	99.6	98.6	99.5	99.4	99.2	99.2	98.3	98.7	98.0	99.2	99.4	99.4	99.6	99.6	99.7	99.4	99.5	99.6	99.7	99.4	1	EF469650
	2	0.3		99.8	99.6	99.3	99.3	99.3	99.1	99.8	98.9	99.8	99.6	99.4	99.4	98.5	98.9	98.2	99.5	99.7	99.7	99.9	99.8	99.9	99.6	99.6	99.8	99.8	99.6	2	EF469651
	3	0.5	0.2		99.5	99.2	99.2	99.1	98.9	99.8	98.9	99.8	99.5	99.3	99.3	98.4	98.8	98.2	99.3	99.7	99.7	99.9	99.7	99.8	99.5	99.5	99.6	99.7	99.5	3	EF469652
	4	0.5	0.4	0.5		99.0	99.0	99.0	98.8	99.6	98.6	99.5	99.4	99.2	99.2	98.4	98.7	98.0	99.2	99.5	99.5	99.7	99.6	99.7	99.4	99.5	99.6	99.8	99.4	4	EF469653
	5	0.9	0.7	0.8	1.0		100	98.7	98.5	99.2	98.3	99.2	99.0	98.8	98.8	97.9	98.3	97.6	98.9	99.1	99.1	99.3	99.2	99.3	99.0	99.0	99.2	99.2	99.0	5	EF469654
	6	0.9	0.7	0.8	1.0	0.0		98.7	98.5	99.2	98.3	99.2	99.0	98.8	98.8	97.9	98.3	97.6	98.9	99.1	99.1	99.3	99.2	99.3	99.0	99.0	99.2	99.2	99.0	6	EF469659
	7	0.8	0.6	0.7	0.8	1.2	1.2		98.5	99.2	98.3	99.1	98.9	99.4	98.8	97.9	98.4	97.6	98.8	99.1	99.1	99.3	99.2	99.4	98.9	98.9	99.2	99.2	98.9	7	EF469660
	8	0.4	0.1	0.2	0.4	0.7	0.7	0.5		99.8	99.8	97.5	97.3	97.2	97.1	96.1	96.5	95.8	97.9	99.7	99.7	99.9	97.6	99.9	97.3	97.3	97.5	97.5	97.3	8	EU146866
	9	0.4	0.2	0.2	0.4	0.8	0.8	0.6	0.2		100	99.1	98.9	98.7	98.6	97.6	98.0	97.3	98.0	99.7	99.8	99.9	99.1	99.9	98.9	98.8	99.0	99.0	98.9	9	EU146879
	10	0.4	0.2	0.2	0.4	0.8	0.8	0.6	0.2	0.0		97.4	97.2	97.0	96.9	95.9	96.4	95.6	97.8	99.7	99.8	99.9	97.4	99.9	97.2	97.1	97.3	97.4	97.2	10	EU146880
	11	0.5	0.2	0.2	0.5	0.8	0.8	0.7	0.2	0.2	0.2		99.5	99.3	99.3	98.4	98.8	98.1	99.3	99.7	99.7	99.9	99.7	99.8	99.5	99.5	99.6	99.7	99.5	11	EU183323
	12	0.6	0.4	0.5	0.6	1.0	1.0	0.9	0.4	0.4	0.4	0.5		99.1	99.1	98.3	98.7	97.9	99.2	99.5	99.5	99.7	99.5	99.7	99.3	99.2	99.5	99.5	99.3	12	EU183324
	13	0.8	0.6	0.7	0.8	1.2	1.2	0.5	0.5	0.6	0.6	0.7	0.9		98.9	98.0	98.6	97.8	99.0	99.3	99.3	99.5	99.4	99.6	99.1	99.1	99.4	99.4	99.1	13	EU183326
	14	0.8	0.6	0.7	0.8	1.2	1.2	1.1	0.6	0.7	0.7	0.7	0.9	1.1		98.0	98.5	98.0	99.0	99.2	99.2	99.4	99.4	99.4	99.6	98.2	99.3	99.4	99.2	14	EU183327
	15	1.4	1.1	1.2	1.2	1.7	1.7	1.6	1.2	1.2	1.2	1.2	1.3	1.6	1.6		98.9	98.2	97.9	98.1	98.1	98.3	98.4	98.4	98.2	98.7	98.4	98.4	98.2	15	EU183328
	16	1.0	0.8	0.9	1.0	1.4	1.4	1.1	0.7	0.8	0.8	0.9	1.1	1.1	1.2	1.1		98.7	98.3	98.6	98.6	98.8	98.9	98.8	98.7	97.9	98.8	98.9	98.7	16	EU183329
	17	1.7	1.5	1.5	1.7	2.0	2.0	2.0	1.5	1.5	1.5	1.6	1.8	2.0	1.7	1.8	1.3		97.6	97.9	98.0	98.0	98.2	98.1	98.1	99.2	98.1	98.2	97.9	17	EU183332
	18	0.4	0.1	0.2	0.4	0.7	0.7	0.6	0.1	0.2	0.2	0.2	0.4	0.6	0.6	1.2	0.8	1.5		99.7	99.7	99.9	99.4	99.9	99.2	99.2	99.3	99.4	99.2	18	DQ447199
	19	0.6	0.3	0.3	0.5	0.9	0.9	0.7	0.3	0.3	0.3	0.3	0.5	0.7	0.8	1.3	1.0	1.6	0.3		100	99.8	99.7	99.3	99.4	99.4	99.6	99.7	99.4	19	DQ837587
	20	0.6	0.3	0.3	0.5	0.9	0.9	0.7	0.3	0.2	0.2	0.3	0.5	0.7	0.8	1.3	0.9	1.6	0.3	0.0		99.8	99.7	99.7	99.4	99.4	99.6	99.7	99.4	20	DQ837588
	21	0.4	0.1	0.1	0.3	0.8	0.8	0.5	0.1	0.1	0.1	0.1	0.3	0.5	0.6	1.1	0.8	1.5	0.1	0.2	0.2		99.9	99.9	99.6	99.6	99.8	99.9	99.6	21	DQ837589
	22	0.4	0.2	0.3	0.4	0.8	0.8	0.6	0.1	0.2	0.2	0.3	0.5	0.6	0.6	1.2	0.8	1.5	0.2	0.3	0.3	0.1		99.9	99.5	99.5	99.7	99.8	99.5	22	DQ862001
	23	0.3	0.1	0.2	0.3	0.7	0.7	0.4	0.1	0.1	0.1	0.2	0.3	0.4	0.6	1.1	0.7	1.5	0.1	0.3	0.3	0.1	0.1		99.6	99.6	99.8	99.9	99.7	23	EF042622
	24	0.6	0.4	0.5	0.6	1.0	1.0	0.9	0.4	0.4	0.4	0.5	0.7	0.9	0.4	1.4	1.1	1.7	0.4	0.6	0.6	0.4	0.5	0.4		99.3	99.5	99.5	99.3	24	EF441276
	25	0.5	0.4	0.5	0.5	1.0	1.0	0.8	0.4	0.5	0.5	0.5	0.7	0.8	0.9	1.4	1.1	1.8	0.4	0.6	0.6	0.4	0.5	0.4	0.7		99.5	99.6	99.3	25	EF441277
	26	0.4	0.2	0.4	0.4	0.8	0.8	0.6	0.2	0.3	0.3	0.4	0.5	0.6	0.7	1.2	0.9	1.6	0.2	0.4	0.4	0.3	0.3	0.2	0.5	0.5		99.8	99.5	26	EF441278
	27	0.3	0.20	0.3	0.2	0.8	0.8	0.6	0.2	0.2	0.2	0.3	0.5	0.6	0.6	1.2	0.8	1.5	0.2	0.3	0.3	0.1	0.2	0.1	0.5	0.4	0.2		99.5	27	EF441279
	28	0.6	0.4	0.5	0.6	1.0	1.0	0.9	0.4	0.4	0.4	0.5	0.7	0.9	0.8	1.4	1.1	1.8	0.4	0.6	0.6	0.4	0.5	0.3	0.7	0.7	0.5	0.5		28	EF441280
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		

Divergence

Molecular size analysis of H5 genes (ducks)

Table 2 shows differences in *HA* gene sequence length ranging from 1741 to 1704 bp. These were submitted as partial sequences by the US Naval Medical Research unit in Egypt to GenBank (Magdi *et al*. 2007).

Phylogenetic analysis of H5 genes (ducks)

HA genes from teal duck strains were aligned with other H5N1 viruses from Egyptian ducks (6 isolates). The result showed high similarity between ducks' HA genes ranging from 87.1 to 99.6% (**Table 5**).

Phylogenetic analysis (**Fig. 2**) showed that the origin of *H5* sequences of all duck isolates was the common teal duck (*Anas crecca*) isolates (A/teal/Egypt/9885-NAMRU3/2005(H5)) and (A/teal/Egypt/14051-NAMRU3/2005 (*H5N1*)) which were captured in the Nile Delta region of Damietta in December 2005 and the *HA* gene sequence was submitted to GenBank under accession numbers EF042623 and EF042624, respectively.

Saad *et al*. (2007) reported that two common teal ducks

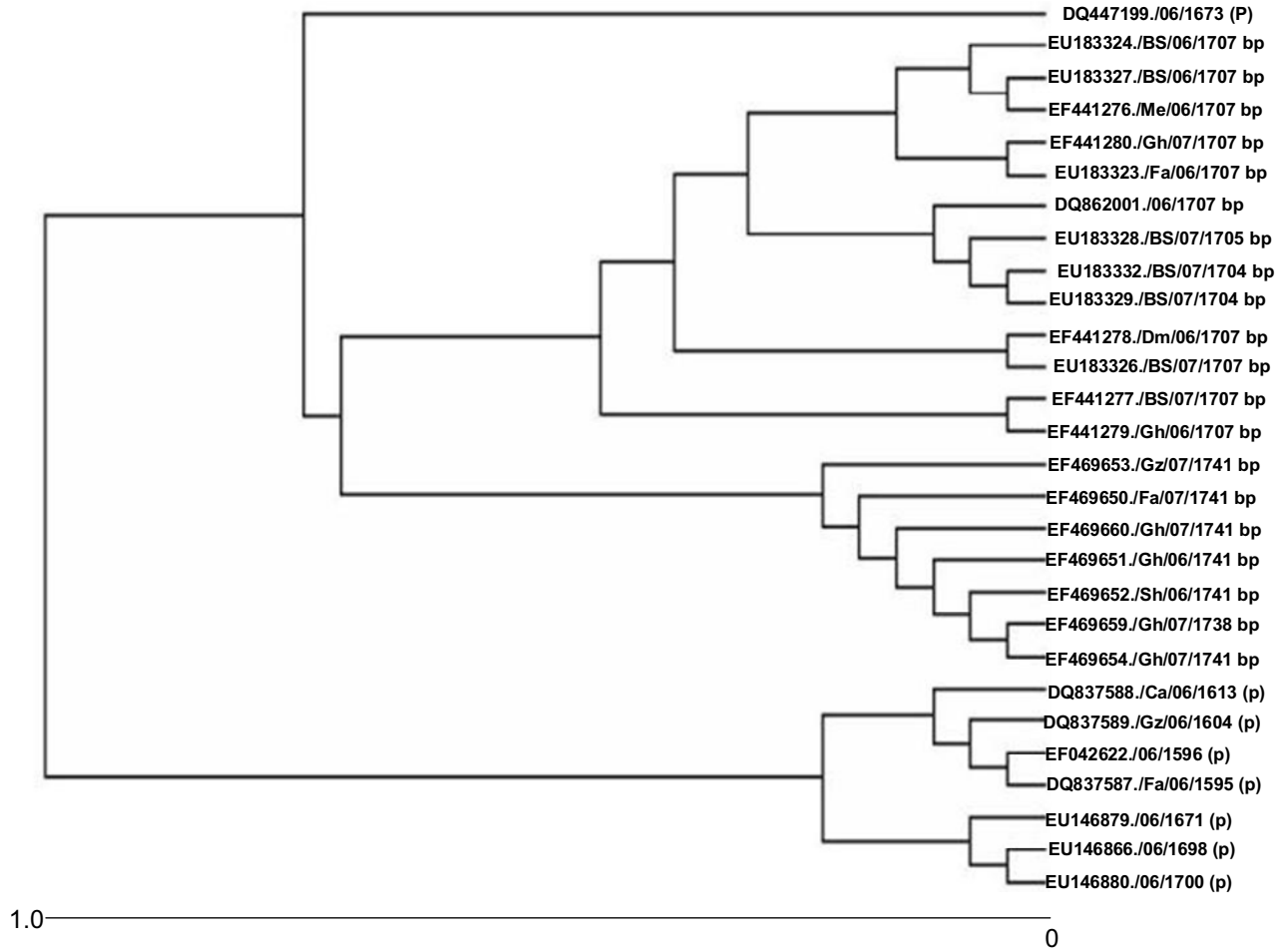

Fig. 1 Phylogenetic tree based on nucleotide sequences of the *Hemagglutinin 5* (*H5*) gene from Egyptian chicken isolates. The tree was generated by the neighbor-joining method in Lasergene 7.1 package (DNASTAR, Madison, Wis.). The tree was rooted to DQ837588 (Ca/06/1613 (p)). NCBI accession numbers/ influenza sequence database number (ISDN) of the viral sequences are shown for references. Dm, Damietta; Mn, Menia; Gh, Gharbiya; Gz, Giza; Bs, Beni-suef; Me, Menofia; Fa, Fayoum; Sh, Sharqiya; Ca, Cairo.

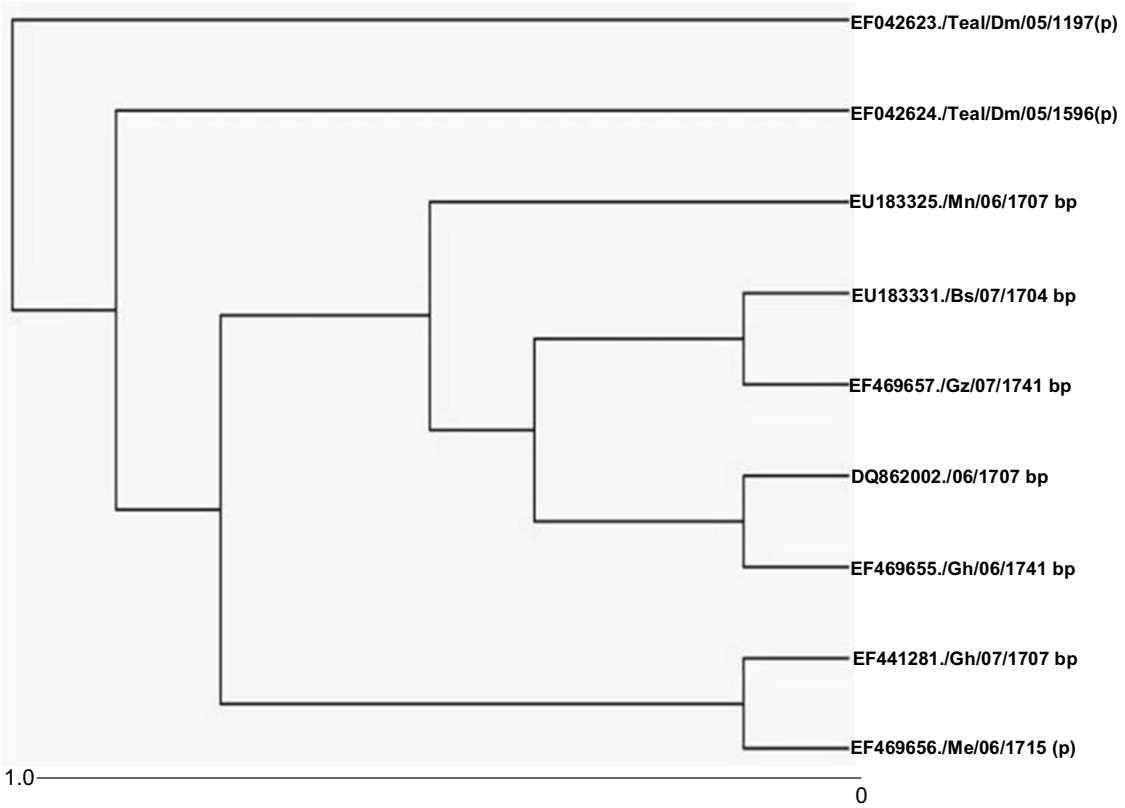

Fig. 2 Phylogenetic tree based on nucleotide sequences of the *Hemagglutinin 5* (*H5*) gene from Egyptian ducks isolates. The tree was generated by the neighbor-joining method in Lasergene 7.1 package (DNASTAR, Madison, Wis.). The tree was rooted to EU183325 (Mn/06/1707 bp). NCBI accession numbers/ influenza sequence database number (ISDN) of the viral sequences are shown for references. Dm, Damietta; Mn, Menia; Gh, Gharbiya; Gz, Giza; Bs, Beni-suef; Me, Menofia.

Table 5 Nucleotide sequence homology of HA gene among Egyptian duck's H5N1 influenza virus

		Percent identity										
		1	2	3	4	5	6	7	8	9		
Divergence	1		88.1	99.0	98.3	99.4	99.1	99.4	99.0	99.2	1	EF042624
	2	11.4		87.9	87.1	88.0	87.7	88.0	87.7	87.7	2	EF042623
	3	0.9	11.8		98.6	99.4	99.2	99.4	99.0	99.2	3	EU183325
	4	1.1	11.9	1.1		98.9	98.7	98.9	98.5	98.9	4	EU183331
	5	0.6	11.7	0.6	0.8		99.5	99.9	99.4	99.6	5	DQ862002
	6	0.8	11.8	0.8	1.0	0.5		99.5	99.6	99.4	6	EF441281
	7	0.6	11.7	0.6	0.8	0.1	0.5		99.3	99.5	7	EF469655
	8	0.9	11.8	0.9	1.1	0.6	0.4	0.7		99.1	8	EF469656
	9	0.7	11.9	0.8	0.8	0.4	0.6	0.5	0.9		9	EF469657
		1	2	3	4	5	6	7	8	9		

Table 6 Nucleotide sequence homology of HA gene among Egyptian human's H5N1 influenza virus.

		Percent identity														
		1	2	3	4	5	6	7	8	9	10	11	12	13		
Divergence	1		99.2	99.1	99.5	98.7	98.8	98.7	98.7	99.2	99.5	99.6	99.3	98.9	1	EF535818
	2	0.8		99.0	99.4	98.6	98.7	98.5	98.6	99.1	99.4	99.5	99.1	98.8	2	EF535819
	3	0.9	1.1		99.3	98.6	98.7	98.5	98.6	98.9	99.3	99.5	99.1	98.8	3	EF535820
	4	0.5	0.6	0.7		99.0	99.0	98.9	99.0	99.4	99.8	99.9	99.5	99.1	4	EF535821
	5	1.3	1.4	1.4	1.0		99.9	99.8	99.8	98.6	99.0	99.0	98.8	98.5	5	EF535822
	6	1.2	1.4	1.4	1.0	0.1		99.9	99.8	98.6	99.1	99.1	98.8	98.5	6	EF535823
	7	1.4	1.5	1.5	1.1	0.2	0.1		99.7	98.5	99.0	99.0	98.7	98.4	7	EF535824
	8	1.2	1.4	1.4	1.0	0.2	0.1	0.2		98.6	99.0	99.0	98.8	98.4	8	EF535825
	9	0.8	0.9	1.1	0.6	1.5	1.4	1.5	1.4		99.3	99.5	99.1	99.5	9	EF535826
	10	0.5	0.6	0.7	0.2	1.0	0.9	1.0	0.9	0.7		99.9	99.6	99.1	10	EU146867
	11	0.4	0.5	0.5	0.1	1.0	0.9	1.0	0.9	0.5	0.1		99.6	99.2	11	EU146868
	12	0.7	0.9	0.9	0.5	1.2	1.2	1.3	1.2	0.9	0.4	0.4		98.8	12	EF061116
	13	1.0	1.1	1.1	0.8	1.4	1.4	1.5	1.4	0.4	0.8	0.7	1.1		13	EF535817
		1	2	3	4	5	6	7	8	9	10	11	12	13		

captured in October 2005 were positive for the *H5* gene based on PCR by specific primers. Sequencing of the *H5* gene showed that this virus was a low pathogenic avian influenza virus (LPAI) which is most closely related to strain A/mallard/Bavaria/1/2005(H5N2) (GenBank accession no. DQ387854).

Phylogenetic analysis of the *H5* gene agreed with the findings of Magdi *et al.* (2007), who showed clustering of the HPAI (H5N1) isolates collected from one geographic region. All HPAI (H5N1) Egyptian human and chicken isolates clustered in one clade with a bootstrap support value of 98%. Furthermore, the H5N1 isolate of A/Teal/Egypt/14051-NAMRU3/2006, which was collected in December 2005, was the common ancestor of the virus isolates which had been isolated in early 2006 in Egypt.

Phylogenetic analysis of *H5* genes (humans)

Multiple sequence alignment of the *H5 HA* genes performed within isolates of infected humans in Egypt revealed that the *HA* genes of H5N1 Egyptian viruses were almost identical to each other (98.6-99.9% identity), as shown in **Table 6**.

Fig. 3 shows the topology of the phylogenetic tree which includes 2 sub-clades which represented the Delta region and Upper Egypt isolates. The tree was evolved from (A/Egypt/2620-NAMRU3/2007(H5N1)) which was isolated from a 5-year-old male from El-Menia governorate with accession number EF535825.

Molecular size analysis of *H5* genes (humans)

Table 3 of the *H5* genes shows that the molecular size of infected human *H5* genes ranged from 1730 to 1713 bp. There is co-linearity between decreasing molecular size and geographical distribution, which means that the molecular size of *H5* genes decreased from the south to the north of Egypt. The sequence length varied from 1741 to 1707 bp (**Table 3**).

According to the WHO's reports about the human mortality in Egypt (updated 24 September 2009), the Ministry of Health of Egypt has reported 87 confirmed cases to date in Egypt, 27 of which were fatal; deaths were concentrated in the Delta region (20/27 dead cases), the remainder in the south of Egypt (7 cases).

The human infection in Aswan may be explained by the transmission of H5N1 from migrated birds which lived at the High Dam Lake in Aswan. So, the avian flu disease may have spread out through the Nile River. Gilbert *et al*. (2008) noted that the movement of infected birds, poultry and pigs manure, in addition to favorable environmental conditions, impacted the ecology of H5N1 viruses which presumably co-evolved with migratory water birds. Gilbert *et al*. (2008) also found that climate change would almost certainly alter bird migration, influence the AI virus transmission cycle and directly affect virus survival outside the host.

The probability of increasing the risk of reassortment or direct mutation into H5N1 viruses to better adaptation of infections from birds (this study) to humans and human-to-human transmission was also reported by Garrett and Fidler (2007) in a case that occurred among members of a family in Sumatra (Indonesia) in 2006.

Phylogenetic analysis between infected human, chicken and duck isolates

The neighbor-joining tree (i.e. phylogenetic tree) based on *HA* gene sequences revealed clustering of the H5N1 viruses into multiple groups that were genetically closely related to each other. We analyzed the Egyptian *H5* genes to test the hypothesis whether the H5N1 virus HAs or infection were derived from H5N1 isolates which were transmitted to Egypt through migrating birds, especially teal ducks. The phylogenetic analysis of *H5* genes isolated from infected chickens, ducks and human (**Fig. 4**) showed that the tree of all Egyptian *H5* genes evolved from two isolates (A/teal/Egypt/9885-NAMRU3/2005(H5) and (A/teal/Egypt/14051-NAMRU3/2005(*H5N1*)) which were isolated from Damietta governorate under accession numbers EF042623 and EF042624, respectively. **Fig. 4** shows that the path of infection started with teal ducks and was then transmitted to

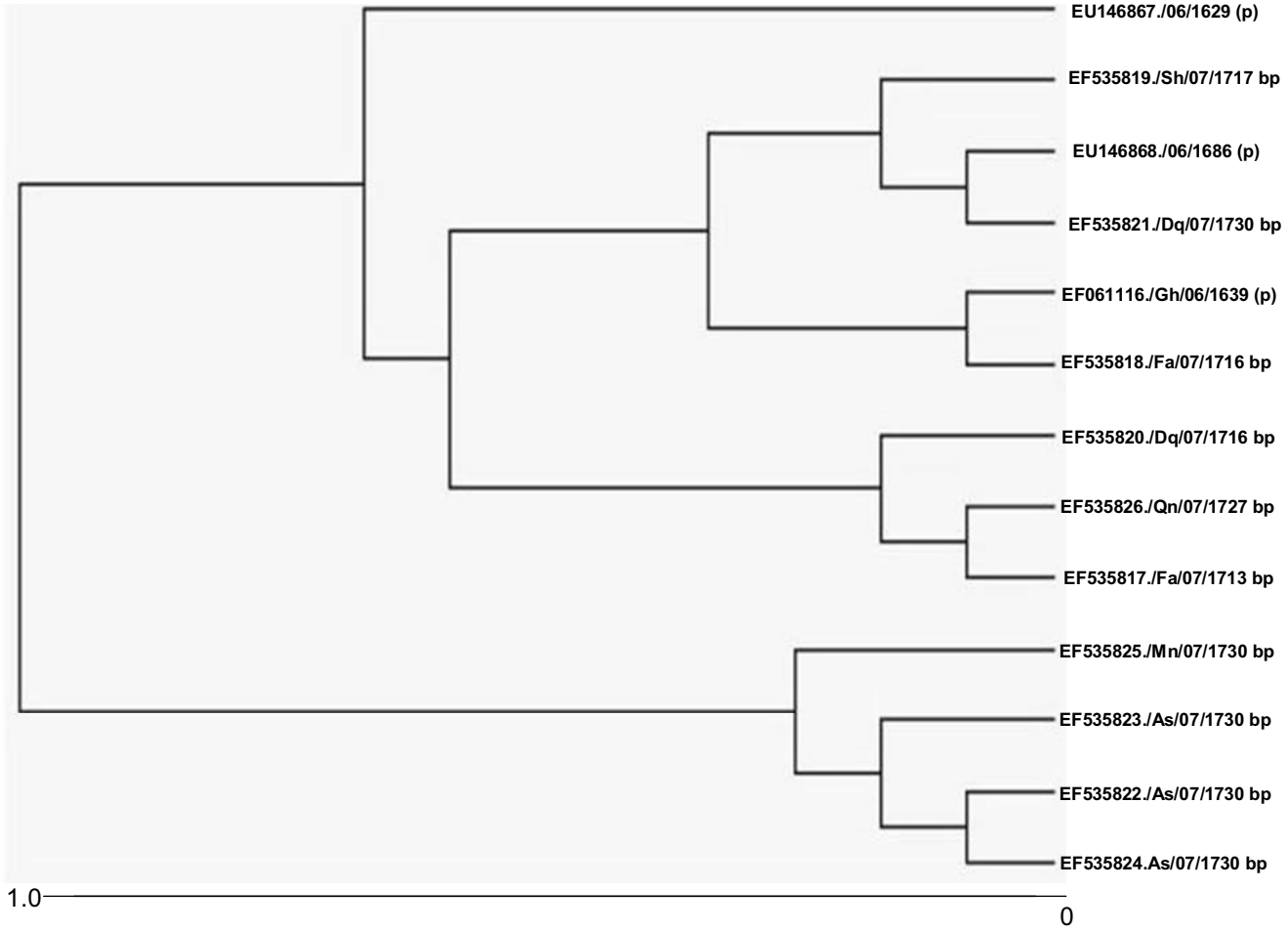

Fig. 3 Phylogenetic tree based on nucleotide sequences of the *Hemagglutinin 5* (*H5*) gene from Egyptian human isolates. The tree was generated by the neighbor-joining method in Lasergene 7.1 package (DNASTAR, Madison, Wis.). The tree was rooted to EF535825 (Mn/07/1730 bp). NCBI accession numbers/ influenza sequence database number (ISDN) of the viral sequences are shown for references. Sh, Sharqiya; Dq, Daqahliya; Fa, Fayoum; Mn, Menia; Gh, Gharbiya; As, Aswan; Qn, Qena.

chickens then on to humans, so we expect that human-to-human transmission in the future will occur rapidly.

Pigs were considered the original "intermediate host" for influenza, because they supported reassortment of divergent subtypes, noticed by the high mutation and reassortment rates of *H5* genes which evolved rapidly, as shown in the phylogenetic trees from 2006 to 2007 (Webster et al. 1992).

Molecular evolution resulted in the existence of a high number of nodes, which reflect an increase in the genetic drift in *H5* genes.

Vaccination strategy

Several strategies are being explored to generate vaccines that will be effective in the event that a new pandemic influenza virus strain emerges in humans. These strategies draw on experience with human influenza vaccines (van den Berg *et al*. 2007).

Neutralizing antibodies are specific to subtype and often strain, so vaccination based on eliciting such antibodies requires accurate prediction of the viral strains that will circulate during the influenza season and leaves little time for vaccine preparation. Even with usual epidemic strains, difficulties and delays in the production of an adequate vaccine supply have occurred in some years.

Previous experience with human influenza virus vaccines has shown that they are effective only if the HA of the vaccine strain and the epidemic strain are antigenically closely matched (Gillim-Ross and Subbarao 2006). Therefore, the choice of vaccine will be based on the antigenic properties of circulating strains and the immunogenic properties of vaccine candidates.

Our studies are a primary step for antigen prediction for vaccine production especially since highly pathogenic H5N1 influenza viruses have continued to evolve since their emergence in 1997, with changes in antigenicity (Horimoto *et al.* 2004).

The choice of the best vaccine against local circulated H5N1 in Egypt will depend on the close antigenetic matching of the HA of the vaccine strain and the epidemic strain. Thus, our suggestion about the candidate strains for vaccine production concentrate on choosing a common ancestor or the root of the phylogenetic tree of the influenza virus gene pool in Egypt as a candidate strain because the vaccines using conserved components of influenza A virus can induce protection against many influenza A strains, including those of divergent subtypes (Epstein *et al*. 2002).

From previous observations we supposed that the most effective pre-pandemic vaccine against local circulated H5N1 in Egypt, chicken viruses should be prepared from sub-clade 3, which evolved from Cairo and Giza isolates (the root of all chicken isolates, accession nos. DQ837588 and DQ837589) while El-Menia isolate (the root of all duck isolates, accession no. EU183325) will be a candidate for a duck vaccine. Finally, the candidate isolate for a human vaccination should be prepared from the El-Menia isolate (the root of all human isolates, accession no. EF535825).

The results may agree with those of the WHO in producing a new H5N1 recombinant vaccine virus which has been developed by the WHO Collaborating Center for the Surveillance, Epidemiology and Control of Influenza at the Centers for Disease Control and Prevention (WHO CC), Atlanta, USA from strain A/Egypt/2321-NAMRU3/2007 which was isolated from a 10-year-old male from Aswan. This strain can be considered the same root of the phylo-

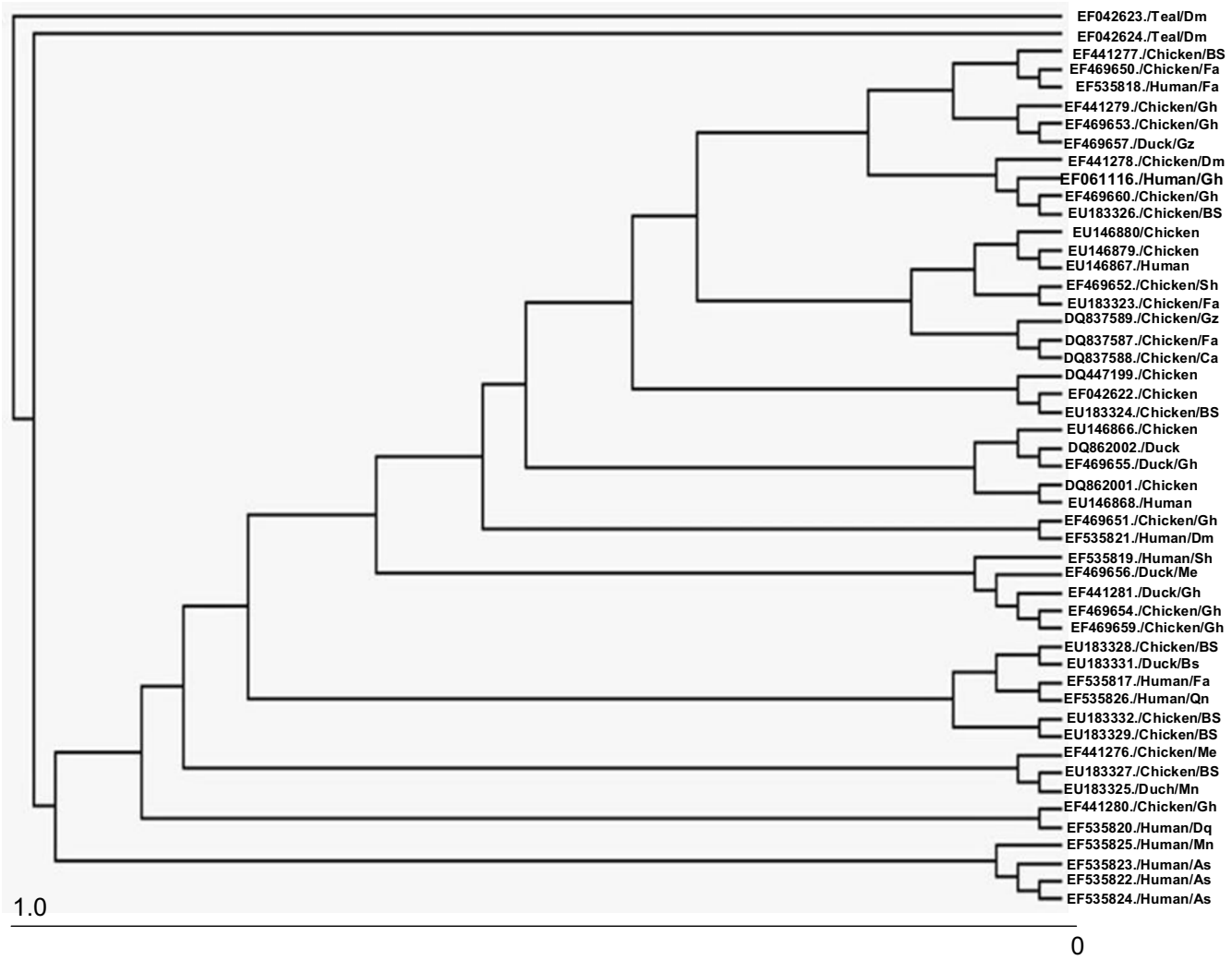

Fig. 4 Phylogenetic tree of H5N1 influenza viruses was constructed through neighbor-joining method in Lasergene 7.1 package (DNASTAR, Madison, Wis) based on sequence distance between each virus genotype of infected chickens, ducks and humans in different geographical regions in Egypt.

genetic tree of human H5N1 (http://www.who.int/csr/disease/avian_influenza/H5N1virus26May/en/index.html).

This study demonstrates the dynamic nature of the influenza virus gene pool in Egypt with continuing gene exchange between the different parts of Egypt. Our findings suggest that the Egyptian H5N1 viruses were likely derived directly from viruses resident in migratory birds. The failure to identify the source of all gene segments and its variants highlight the need for continued and extensive surveillance in both migratory birds and domestic populations in larger regions. Such surveillance is crucial for effective pandemic influenza preparedness.

To effectively control the virus from circulating in poultry and other hosts, an efficient post-vaccination surveillance program should be established. An ideal veterinary vaccine should be safe, potent, thermostable, single dose, easy to administer and allow the differentiation between vaccinated and infected animals (Swayne 2003; Capua and Marangon 2006).

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