Comparative Molecular Analysis of Egyptian Strains of Highly Pathogenic Avian Influenza (HPAI) Virus H5N1 Isolated During 2006 And 2007

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Abstract: Isolated strains of HPAI H5N1 during February 2006 from infected chicken located at El-Qanater El-Khiria center, Qaluobia, Egypt were molecularly characterized and compared with those isolated strains during January 2007 from the same locality. The isolated strains from infected chicken during 2006 were closely related to strain isolated from turkeys located in Turkey at 2005 **(A/ty/Turkey/1/05)** and to **A/Vietnam/1194/04** strain isolated from human cases of AI H5N1 and induced higher case fatality in human in Vietnam. The phylogenetic analysis of HA and NA genes of the isolated strains during 2006 revealed that, these isolates are located in Eurasia- African lineage which contains AI H5N1 strains from Egypt, Turkey, Romania, Iraq, Mongolia, Iran, Korea and Nigeria and differed from those isolated in South-Eastern of Asia which contains Cambodia, Thailand, and Vietnam Nam strains of AI H5N1. The phylogenetic analysis of HA, NA, and M genes of Egyptian isolates of January 2007 from the same locality showed minor antigenic variation from those of 2006 and located in a new sub-clade from Egyptian strains of 2006 and all of them located in Euro-African lineage. All 8 genes were closely related to the genes of onther Egyptian H5N1 isolates from chicken, duck, and human. The sequence of NAs did not possess any know oseltamivir resistance mutations. Also the M2 sequences did not possess amantadine resistance mutations.
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1. Introduction

The first wave of HPAI H5N1 strains in Egypt had been appeared in February 2006 and extended to June 2006, then the disease was subsided from the end of this month till the end of August, after that the second wave had been started in September 2006 and still to this day in our country. The HPAI H5N1 viruses are now endemic in many Eurasia and African countries, resulting in repeated outbreaks in poultry and increased cases of human infection. The immediate precursor of these HPAI viruses is believed to be A/goose/Guangdong/1/96(Gs/GD) like H5N1 HPAI viruses first detected in Guangdong, China, in 1996. Their host range had extended from goose to domestic ducks, which played a key role in the genesis of the 2003/04 outbreaks. Phylogenetic analyses revealed that Gs/GD-like virus was likely derived from an LPAI H5 virus in migratory birds. However, its variants arose from multiple reassortments between Gs/GD-like virus and viruses from migratory birds or with those Eurasian viruses isolated in the 1970s **(Simsetal., 2005 and Duan etal., 2007,).** Another study revealed that, the origin of first Asian HPAI H5N1 virus in Guangdong by the reassortment of Nanchang (close to Guangdong) and Hokkaido (Japan) (H1N1-55 and H5N3-51) viruses **(Mukhtaretal., 2007).** The H6N1 virus is the first known isolate with seven H5N1-like segments and may have been the donor of the neuraminidase and the internal genes of the H5N1 viruses. The high homology between the internal genes of H9N2, H6N1, and the H5N1 isolates indicates that these subtypes are able to exchange their internal genes and are therefore a potential source of new pathogenic influenza **(Hoffmann, et al., 2000).** Phylogenetic analysis of 7 influenza (H5N1) viruses isolated from poultry in Western Siberia and the European part of the Russian Federation during July 2005-February 2006 showed high homology to Qinghai-like influenza (H5N1) viruses; a close genetic relationship between the H5N1 strains isolated from poultry and wild migratory waterfowls and also suggested genetic reassortment among the analyzed isolates **(Lipatov*et al.*, 2007)**. Phylogenetic analyses of H6N2 chicken viruses indicated that the H6N2 most likely arose from a reassortment between two South African LPAI ostrich isolates: an H9N2 virus isolated in 1995 and an H6N8 virus isolated in 1998. It is probable that the ostrich H6N8 and H9N2 progenitors of the chicken H6N2 viruses were introduced to ostriches by wild birds **(Abolnik *et al.*, 2007 a&b)**. The H9N2 strain that was isolated from quails and H5N1 subtype viruses isolated from chicken have also exchanged gene segments to generate currently circulating reassortants of both subtypes that have pandemic potential **(Xu*et al.*, 2007).** The Italian HPAI viruses

arose from low pathogenicity strains, and that a deletion in the NA stalk followed by the acquisition of additional glycosylation near the receptor binding site of HA1 may be an adaptation of H7 viruses to a new host species i.e. domestic poultry **(Banks *et al.*, 2001).** Thus, genetic reassortment between avian and human influenza strains does occur in the emergence of pandemic and inter-pandemic influenza A viruses **(Shu*et al.*, 1996).**The present study aimed to isolate and identify the circulated HPAI virus in year 2007 followed by genotyping and comparing of the isolated strains with those isolated from the same locality during 2006 and other worldwide HPAI strains

2. Material and methods

Sample collection:

Oropharyngeal and cloacal swabs were collected in January 2007, from affected chicken reared in backyards in El- Qanater El- Khiria Center, Qaluobia; in viral transport media (HBSS with isotonic ph 7-7.2 contained penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50µg/ml) and mycostatin (1000 units/ml) for oropharyngeal swabs but at five-fold higher concentrations for faeces and cloacal swabs. Collected swab suspensions were stored in ice box, then transported to WHO National Influenza Center (WHONIC) for diagnosis of influenza viruses, VACSERA, Agouza, Egypt, within 24 hours

Virus isolation and identification:

Virus isolation and identification were done according to the protocol of OIE manual 2005 following standard bio-safety guidelines of WHO **(**OIE 2005**),** the virus was isolated from collected swabs immersed in HBSS, the swabs were centrifuged at 3000 rpm and 0.2 ml were inoculated in each of SPF embryonated chicken eggs (ECE), inoculated SPF eggs were incubated at 35-37°C and all eggs remaining at the end of the incubation period, were chilled to 4°C, the allantoic fluid was aseptically collected and tested for heamagglutination (HA) activity using 0.5% Chicken RBCs.

Antigenic analysis:

The isolated strains were identified in WHONIC, VACSERA, Agouza, in Egypt using HI influenza Kit contained reference influenza type A antisera against human (H1N1- A/NC/20/99 A/New Caledonia; H3N2- A/Panama/2007/96) human strain; and avian influenza virus strains (A/H5(H5N1): chicken strain). The isolated and identified strains in Egypt were sent to WHO Calibrating Center (WHOCC), London, UK, for antigenic analysis by HI influenza Kit contained reference influenza type A antisera against many human (**A/Hong Kong/156/97**; **A/Hong Kong/213/03**; **A/Vietnam/1194/04**; **A/Indonesia/5/05**) and avian influenza virus strains (**Ck/Scotland/59**; **Qu/Cirebon/BBVet/05**; **ty/Turkey/1/05**).

Phylogenetic analysis:

The identified virus strains were subjected for phylogenetic analysis in WHOCC influenza reference Lab., at London **Data collection:** data were collected from presented reports by the Egyptian authorities (General Organization of Veterinary Services GOVSs, Ministry of Agriculture and reclamation of lands, Ministry of Health and population MOH, and State Information Services SIS) during the outbreak **(www.birdflu.sis.gov.eg (Statement of Supreme National Committee to Combat Bird Flu, 3/1/2008)**.

3. Results

Table (1) Comparison between affected cases from 31/8/2006* to 31/12/2007

Year	Type of cases					
	Birds			Human		
	Farms	Houses birds	Zoo cases	Diseased	Died	% Case fatality
2006	845	266	5	18	10	55.5
2007	017	058	0	25	09	36.0
Total	862	324	5	43	19	44.2

* The second phase of HPAI was appeared in the autumn and winter months from September 2006 after disappearance of any recorded cases from June to August 2006

Table (2) Antigenic analysis of avian influenza viruses by (HI) in WHONIC Egypt

Tested samples	Reference control positive anti influenza viruses type A sera		
	H1N1* A/NC/20/99	H3N2** A/Panama/2007/96	H5N1***
Reference Control positive antigens			
A/H1*	5120	160	40
A/Panama/2007/96*	0	5120	0
A/H5*	0	0	640
Tested sample 2007			
ck/Egypt/1/07 N	0	0	320
ck/Egypt/2/07 C	0	0	320

*A/H1 (H1N1) A/New Caledonia) human strain *** A/H5(H5N1): chicken strain
**A/H3 (H3N2) A/Panama/2007/96*: human strain

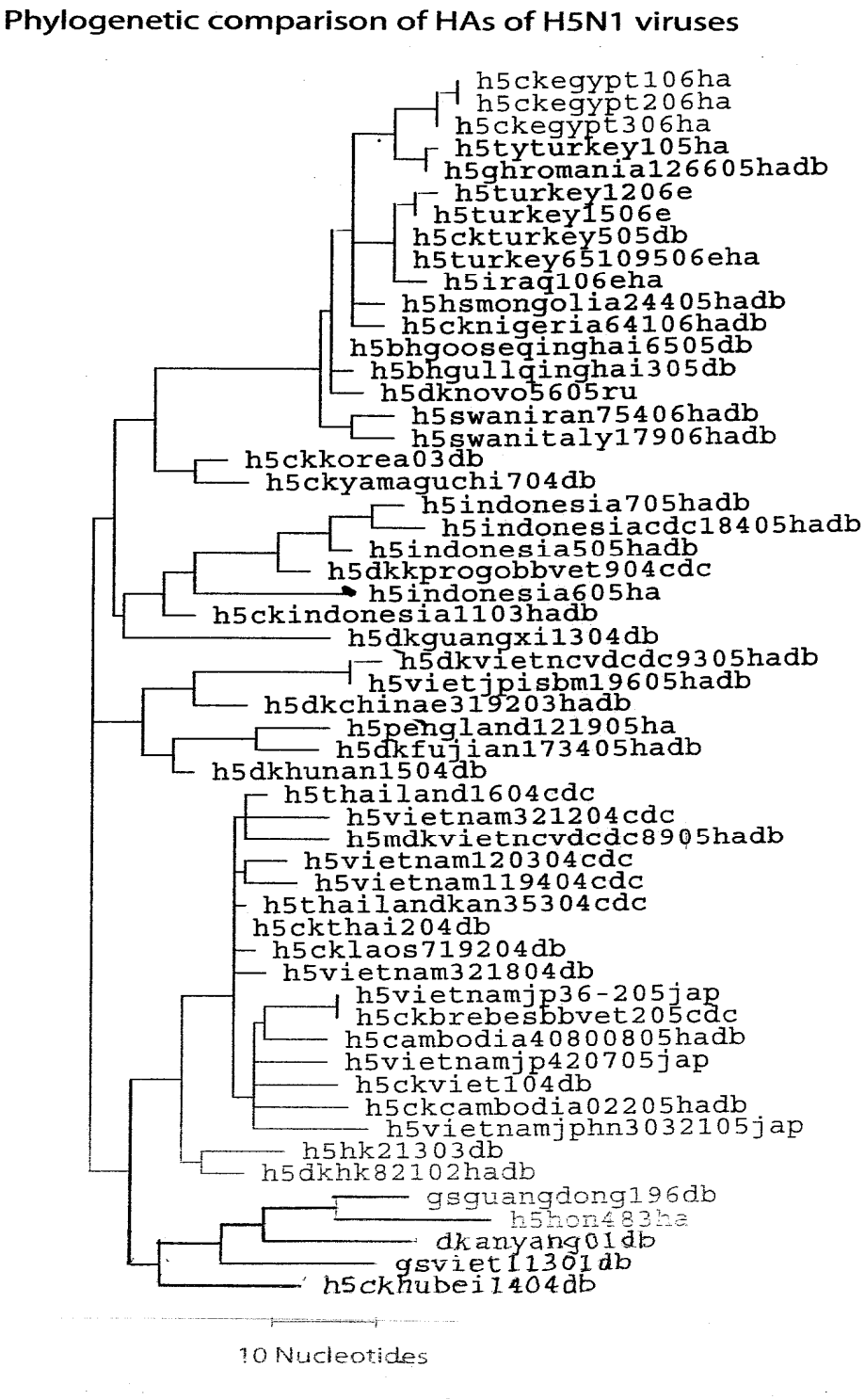

Fig. (1) Phylogenetic comparison of H5 HA genes of outbreaks 2006

Fig. (2) Phylogenetic comparison of H5 NA genes of outbreaks 2006

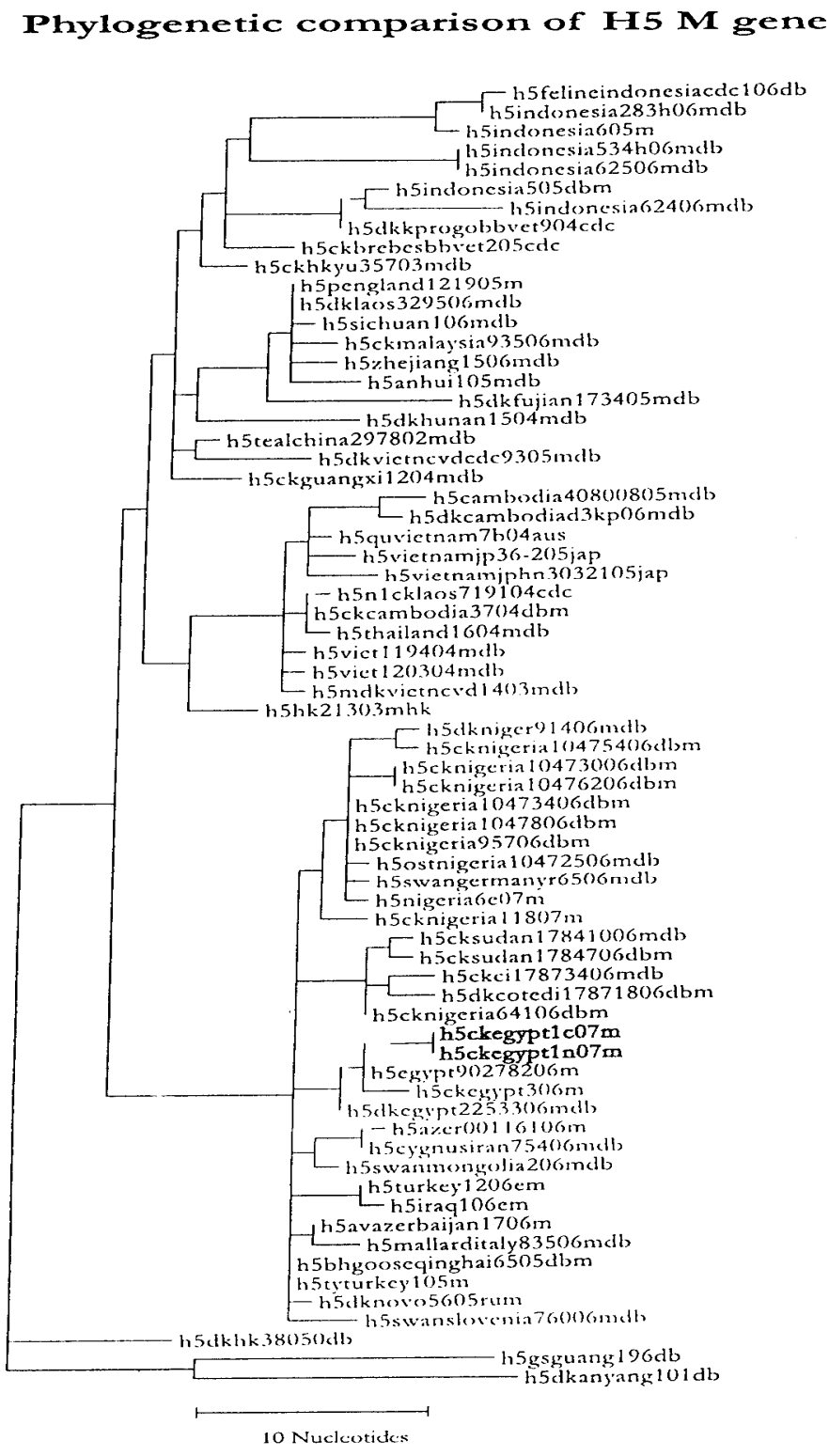

Fig.(3) Phylogenetic comparison of AI H5 M genes of outbreaks 2007. The isolated strains put in Euro- African lineage

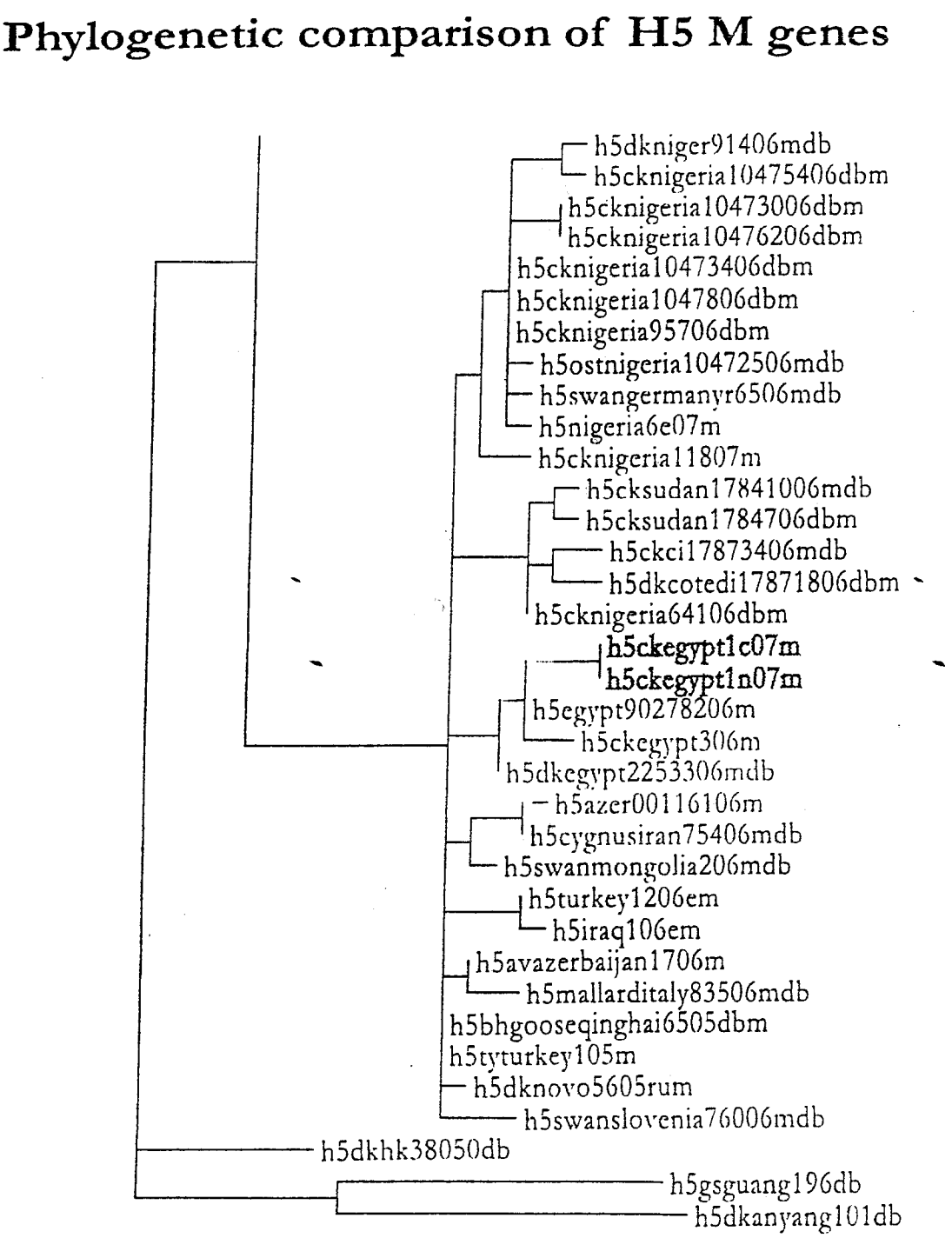

Fig.(4) Phylogenetic comparison of AI H5 M genes of outbreaks 2007. The isolated strain during 2007 put in new sub- clade from those isolated during 2006

Fig.(5) Phylogenetic comparison of AI H5 HA genes of outbreaks2007

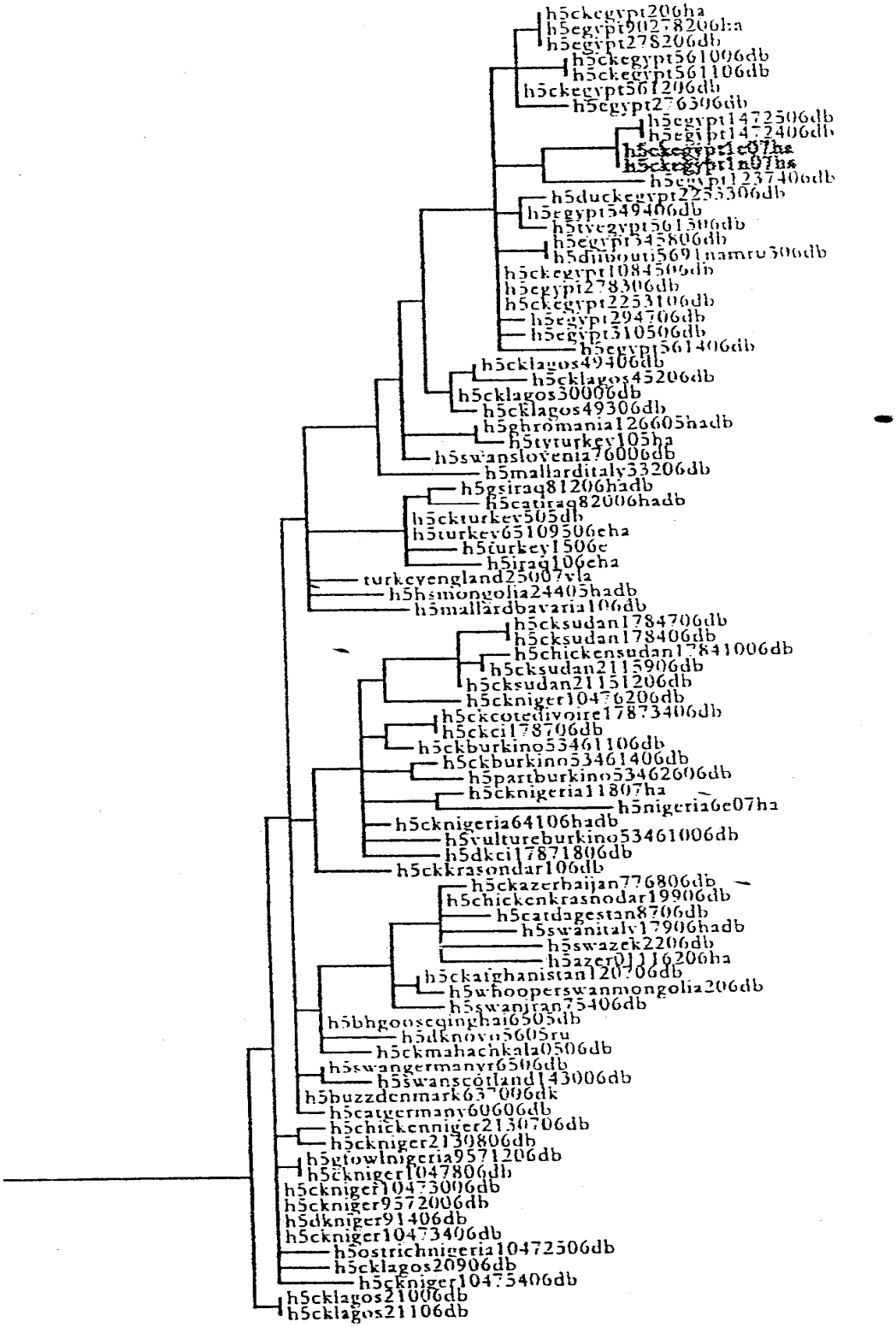

Fig.(6) Phylogenetic comparison of AI H5 HA genes of outbreaks 2007

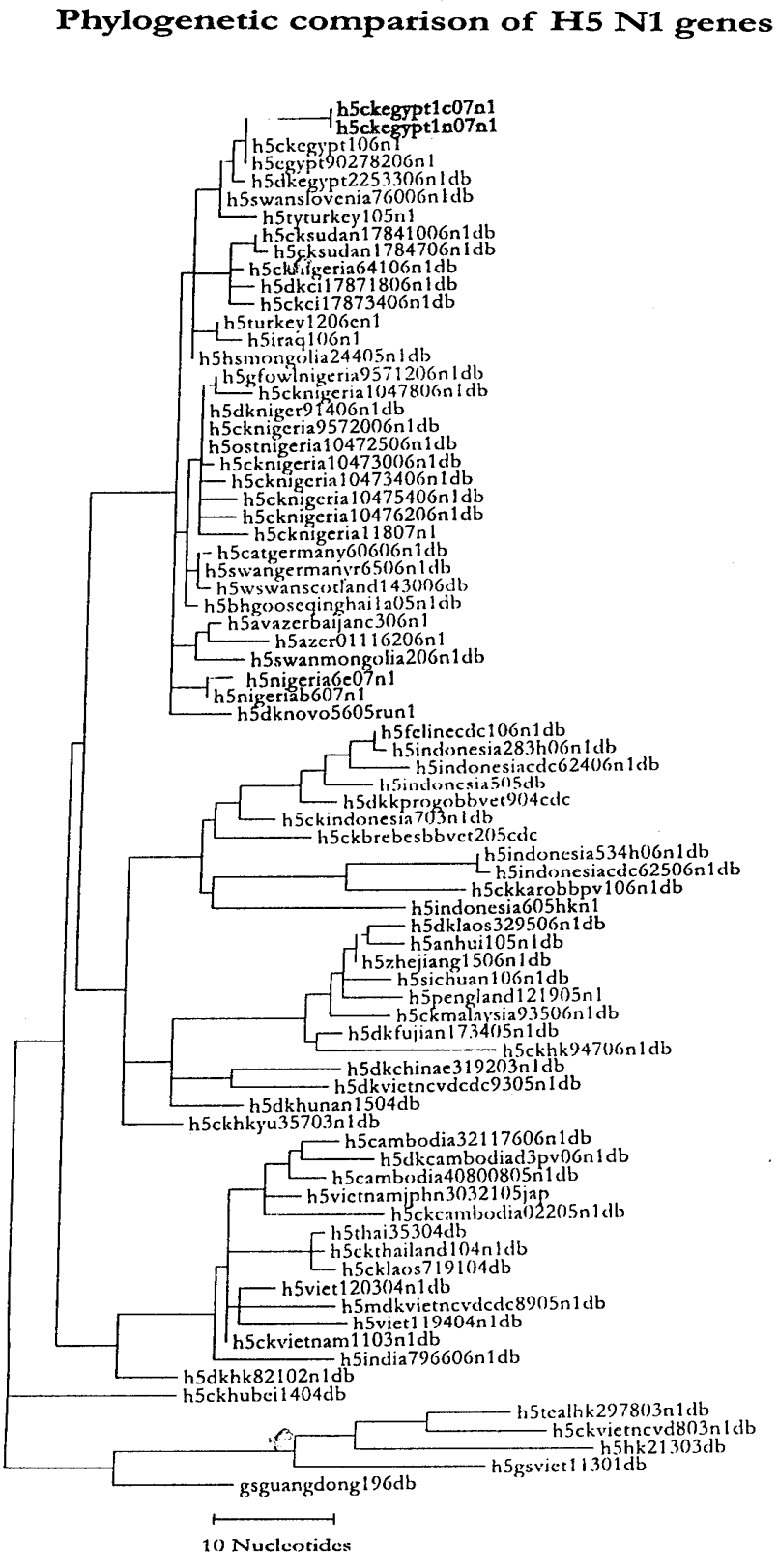

Fig.(7) Phylogenetic comparison of AI H5 NA genes of outbreaks 2007

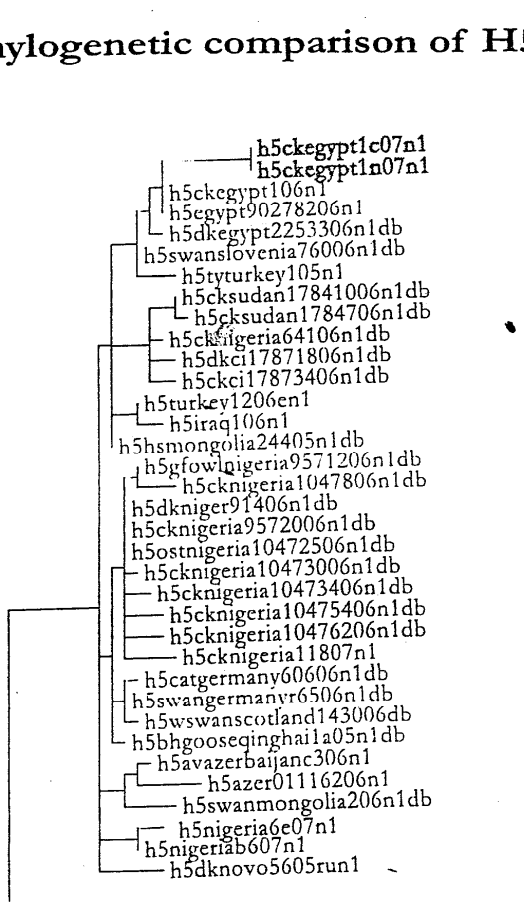

Fig.(8) Phylogenetic comparison of AI H5 NA genes of outbreaks 2007

4. Discussion

Since the HPAI H5N1virus was first spotted in Egypt on February 2006, the government has been exerting every possible effort to contain it. Though bird flu was contained to a great extent, some cases continue to be detected especially among home-raised chickens. In the second phase of bird flu in Egypt (extended from autumn months 2006 to winter months 2007) it was noticed that it appears mostly in the household and domestic poultry rearing but to a less extent in poultry farms. A campaign was launched by the Ministry of Agriculture nationwide to a free charged vaccination of the household poultry according to a time table as about 86 million doses will be used to vaccinate two million poultry by day, the owners of vaccinated poultry will be given a special card indicating date of vaccination and if they refuse, their poultry will be culled immediately **(**www.birdflu.sis.gov.eg/html/flu01021111.htm ((Statement of Supreme National Committee to Combat Bird Flu, 3/1/2008)**).** Although about 60 per cent of poultry reared domestically has been inoculated, the disease still appears day by day and not completely eradicated from Egypt. The number of bird flu-affected farms has decreased from 845 at June 2006 to 17 at the end of 2007 and few of these farms applied vaccination against H5N1 HPAI. Therefore, several challenges are still facing Egypt in fighting the H5N1 virus, mainly home-raised poultry, as most families are still reluctant to vaccinate the birds they raise. The reasons for spreading bird-flu disease once again in vaccinated flocks may be due to the way of preserving and keeping the vaccines or mutation of the circulated field virus since February 2006.This study investigates the current status of bird flu in Egypt with special refer to the circulating virus

Figures representing poultry infection in 2007 had been receded in comparison with those of 2006 (Table 1). In 2006, 845 farms, 266 cases of domesticated poultry and 5 zoo cases had been affected by the HPAI H5N1 virus but in 2007, only 17 farms and 58 cases of domesticated poultry have been affected and no zoo cases have been reported so

far. The lowest figure of affected farms at 2007 than that of 2006 denotes to the effective measures of the vaccination campaign program. On the other hand, the figures of human cases in 2006 was 18 diseased with 10 deaths and case fatality 55.5% but it was 25, 9, and 36% for diseased, deaths and case fatality percentage respectively in 2007(www.birdflu.sis.gov.eg/html/flu01021111.htm (**Statement of Supreme National Committee to Combat Bird Flu, 3/1/2008**).The isolated strains during 2007 was diagnosed as H5N1 in WHO National Influenza Center (WHONIC) for diagnosis of influenza viruses, VACSERA, Agouza, Egypt,(Table 2). The identified isolates were confirmed as H5H1 at in WHOCC influenza reference Lab., at London (Table 3)

The reasons for spreading the disease in vaccinated flocks are questionable. The emerge of new strains of HPAI from the circulated ones is considered a characteristic feature of HPAI either by antigenic shift or drift. The antigenic drift is occurred as a result of point mutation by a change in one nucleotide resulting in change in one amino acid of the protein chain in order. Comparison between more than 3000 RNA sequences of segment 8 of type A influenza viruses a unique single nucleotide substitution typically associated with recent H5N1 strains was found and this explain the aggressive recent outbreaks of H5N1 strains**(Lipatovetal., 2007).** It is very important to mentioned that, the wide spread of HPAI since 2003 to about 60 countries all over the world make the AI virus as a plural virus as between December 2003 and January 2004 highly pathogenic avian influenza (HPAI) H5N1 infections of poultry were declared in China, Japan, South Korea, Laos, Thailand, Cambodia, Vietnam, and Indonesia. In 2004 an outbreak was reported in Malaysia. In 2005 H5N1 outbreaks were recorded in poultry in Russia, Kazakhstan, Mongolia, Romania, Turkey, and Ukraine, and virus was isolated from swans in Croatia. In 2004 HPAI H5N1 virus was isolated from smuggled eagles detected at the Brussels Airport and in 2005 imported caged birds held in quarantine in England. In 2006 HPAI was reported in poultry in Iraq, India, Azerbaijan, Pakistan, Myanmar, Afghanistan, and Gazza in Asia; Albania, France, and Sweden in Europe; and Nigeria, Cameroon, and Niger in Africa; as well as in wild birds in some 24 countries across Asia and Europe. In 2003, over 25,000,000 birds were slaughtered because of 241 outbreaks of HPAI caused by virus of H7N7 subtype in the Netherlands. The virus spread into Belgium (eight outbreaks) and Germany (one outbreak). HPAI H5N2 virus was responsible for outbreaks in ostriches in South Africa during 2005. HPAI H7N3 virus was isolated in Pakistan in 2004. Low-pathogenicity avian influenza (LPAI) H5 or H7 viruses were isolated from poultry in Italy (H7N3 2002-2003; H5N2 2005), The Netherlands (H7N3 2002), France (H5N2 2003), Denmark (H5N7 2003), Taiwan (H5N2 2004), and Japan (H5N2 2005). Much isolation of LPAI viruses of other subtypes were reported from domestic and wild birds. Infections with H9N2 subtype viruses **(Alexander, 2007**).

To better understand the ecology and epidemiology of the circulatory HPAI virus in our country, we sequenced and analyzed the hemagglutinin (H), neuraminidase (N), and matrix (M) genes of the isolated influenza A (H5N1) viruses collected from infected chicken located in El-Qanater El-Khiria, Qaluobia governorate, Egypt during outbreak 2006 and 2007 and comparing these isolates with those isolated from Europe, northern Africa, and southeastern Asia.

The phylogenetic analysis of H and N genes of isolated HPAI H5N1 revealed that, the Egyptian strains that isolated in 2006 from El-Qanater El-Khiria Center, Qaluobia**(h5ckegypt 106 ha , h5ckegypt 206 ha , h5ckegypt 306 ha / h5ckegypt 106 n , h5ckegypt 106 n , h5ckegypt 106 n1)**, are closely related to H5N1 strain isolated from turkeys located in Turkey at 2005 (**A/ty/Turkey/1/05)** influenza strain and toH5N1 strain isolated from human cases in Viet Nam at 2004 **(A/Vietnam/1194/04)** and were located in Eurasia-African lineage as in (Fig.1&2).

Comparison of H5 M genes of Egyptian strains 2007 **(h5ckegypt 1c 07m, h5ckegypt 1n 07m)** and other isolates all over the world revealed that, all worldwide isolates are lies in three distinct lineages, the first one contains Euro-African AI H5 strains; the second contains the south eastern Asian strains, and the third contains Indonesia Strains and Asian/Russian/European strain, and all of these lineages originate from 0 lineage of strains isolated from geese located in Guangdong 1996 and ducks in Hong kong 1997 (Fig. 3).The newly isolated strains from El-Qanater El-Khiria 2007 were located in a new sub-clade from the isolated strain from the same locality 2006(**h5ckegypt306m**)and all Egyptian strains were locate in a sub-clade from those isolated from Africa and Aurasia strains(Fig.3).

On the basis of H5 HA genes analysis of Egyptian isolates during 2007 (**h5ckegypt 1c 07ha, h5ckegypt 1n 07ha)** in comparison with those of African strains and worldwide strains it revealed that, all African strains clustered in the Euro-African lineage within three sub lineages denominated A (south-west Nigeria, Egypt, Djibouti), B (south-west Nigeria, Niger) and C (northern Nigeria, Burkina Faso, Sudan, Côte d'Ivoire), with distinct geographical distributions within Africa. Probable

non-African ancestors within the west Asian/Russian/European lineage distinct from the south-east Asian lineages were identified for each sub lineage (Fig. 4) and these obtained results are in agreement with those obtained by **(Duan*et al.*, 2007, Ducatez *et al.*, 2007 a , Salzberg *et al.*, 2007, and Zohari *et al.*, 2008).**

Moreover, the phylogenetic comparison of H5 N1 genes of these isolated strains (**h5ckegypt 1c 07n1, h5ckegypt 1n 07n1)** revealed the same lineages (Fig. 5)

The phylogenetic analysis of Egyptian strains isolated in 2007 (**h5ckegypt 1c 07m, h5ckegypt 1n 07m /or h5ckegypt 1c 07ha, h5ckegypt 1n 07ha / or h5ckegypt 1c 07n1, h5ckegypt 1n 07n1)** in bold words in Fig. 3-5) revealed that the Egyptian isolates of 2007 showed some antigenic minor variation from those isolated in 2006 (**(h5ckegypt 106 ha , h5ckegypt 206 ha , h5ckegypt 306 ha / h5ckegypt 106 n , h5ckegypt 106 n , h5ckegypt 106 n1)**, as it put in subclade from those isolated at 2006 from the same locality in Egypt and this declaration shows minor antigenic variation and run in a parallel line with those results obtained by **(Gultyaev *et al.*, 2007)** who revealed that, the recent outbreaks of avian influenza are being caused by unusually virulent H5N1 strains which become more aggressive than previously circulating strains. And after they had been compared more than 3000 RNA sequences of segment 8 of type A influenza viruses they found a unique single nucleotide substitution typically associated with recent H5N1 strains.

On conclusion, the highly pathogenic avian influenza isolates all over the world falls into 3 distinct lineages, 1, 2 and 3. One of them contains all known non-Asian isolates (Euro-African lineage) and the second lineages contain the most Asian/Russian/European isolates. While the third contains the South East Asian strains isolated from Cambodia, Vietnam, and Thailand which was predominant in the early phase of the outbreaks and does not yet have any circulating subclades. Asian/Russian/European AI strains had been isolated from Indonesia, China, Laos, Hong Kong, Malaysia & England. The highly virulent strain, which has caused a high rate of human infection and also a high mortality rate among humans in Indonesia, however, seems to be an entirely separate strain, classified as sublineage, and the region has become endemic to this strain. The increase in worldwide human cases was, in part, owing to the spread of the viruses across Eurasia and Africa. The origin of all these strains can be traced to lineage 0 from Hong Kong, where bird flu was first detected in 1997.

The phylogenetic analysis of complete genomes of influenza (H5N1) viruses isolated from El-Qanater El-Khiria, Qaluobia, Egypt, clearly depict the lineages now infecting wild and domestic birds in Europe and Africa and show the relationships among these isolates and other strains affecting both birds and humans (Fig.1-5). The new Euro-African lineage which was the cause of several recent (2006) fatal human infections in Egypt and Iraq has been split into 3 distinct, independently evolving sub-lineages(subclades). A, B, and C (Fig.2-4)

On the other hand, all 8 genes of these isolates were closely related to the genes of other Egyptian H5N1 isolates from chicken, duck, and human. The sequence of NAs did not possess any known oseltamivir resistance mutations. Also the M2 sequences did not possess amantadine resistance mutations as declared by the WHOCC, London, UK.

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