

Comparative Molecular Analysis of Isolated Strains of Highly Pathogenic Avian Influenza Virus H5N1 during 2006 and 2007

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Highly pathogenic avian influenza (AI) H5N1 strains have been isolated during February 2006 from infected chicken located at El-Qanater El-Khiria center, Qaluobia, Egypt. These isolates were molecularly characterized and compared with those H5N1 strains isolated during January 2007 from the same locality. The strains isolated from infected chicken during 2006 were closely related to A/ty/Turkey/1/05 strain which had been isolated from turkeys in Turkey 2005 and to A/Vietnam/1194/04 strain isolated from human cases of AI H5N1 and induced higher case fatality in human in Viet Nam. The phylogenetic analysis of haemagglutinin (HA) and neuraminidase (NA) genes of 2006 strains revealed that these isolates are located in Euro-Asio-African lineage which contains AI H5N1 strains from Egypt, Turkey, Romania, Iraq, Mongolia, Iran, Korea and Nigeria and differed from those strains isolated in South-Eastern of Asia which contains Cambodia, Thailand, and Viet Nam AI H5N1 strains. The phylogenetic analysis of HA, NA, and Matrix (M) genes of Egyptian isolates of January 2007 from the same locality showed minor antigenic variation from those of 2006 and have been located in a new sub-clade from the Egyptian strains of 2006 and all of them are located together in the Euro-African lineage.

Key Words: Highly Pathogenic Avian Influenza Virus H5N1 – Phylogenetic Analysis – HA – NA – Euro-African Lineage.

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Calibrating Center (WHOCC), London, UK, for antigenic analysis by HI influenza Kit contained reference influenza type A antisera against many human and avian influenza virus strains.

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 (16).

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RESULTS AND DISCUSSION

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

Year	Type of cases					
	farms	Birds Houses birds	Zoo cases	diseased	human 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*	H3N2**	H5N1***
	A/NC/20/99	A/Panama/2007/96	
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/H3 (H3N2) A/Panama/2007/96*: human strain

*** A/H5(H5N1): chicken strain

Phylogenetic comparison of HAs of H5N1 viruses

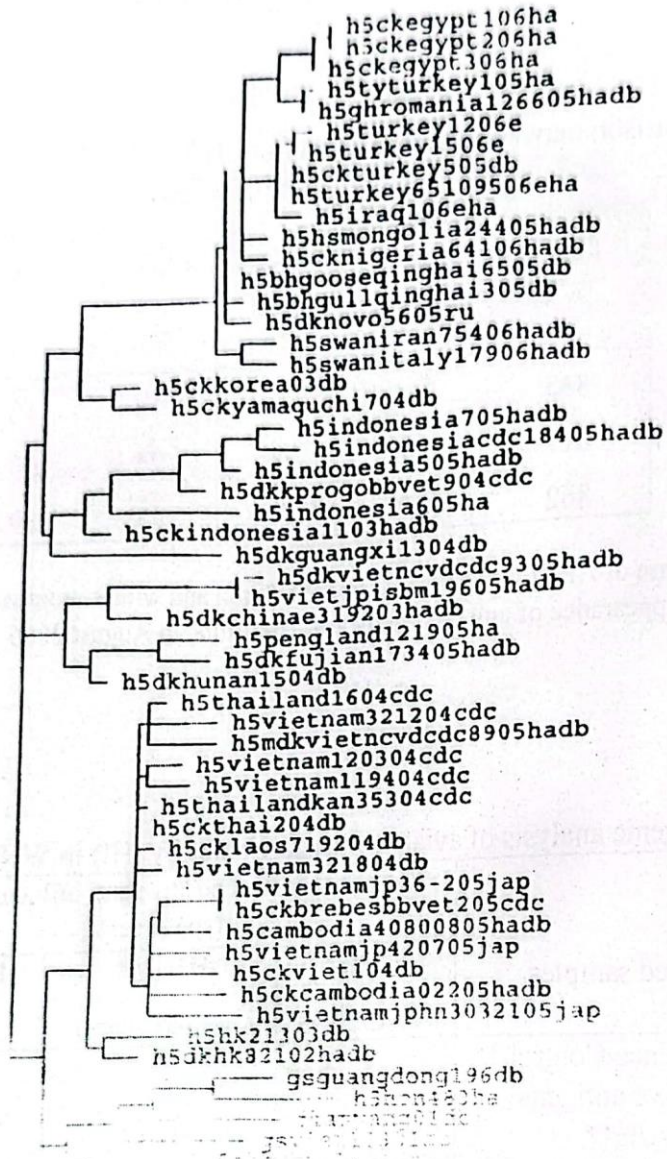


Fig. (1) Phylogenetic comparison of H5 HA genes of outbreaks 2006

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Phylogenetic comparison of NAs of H5N1 viruses

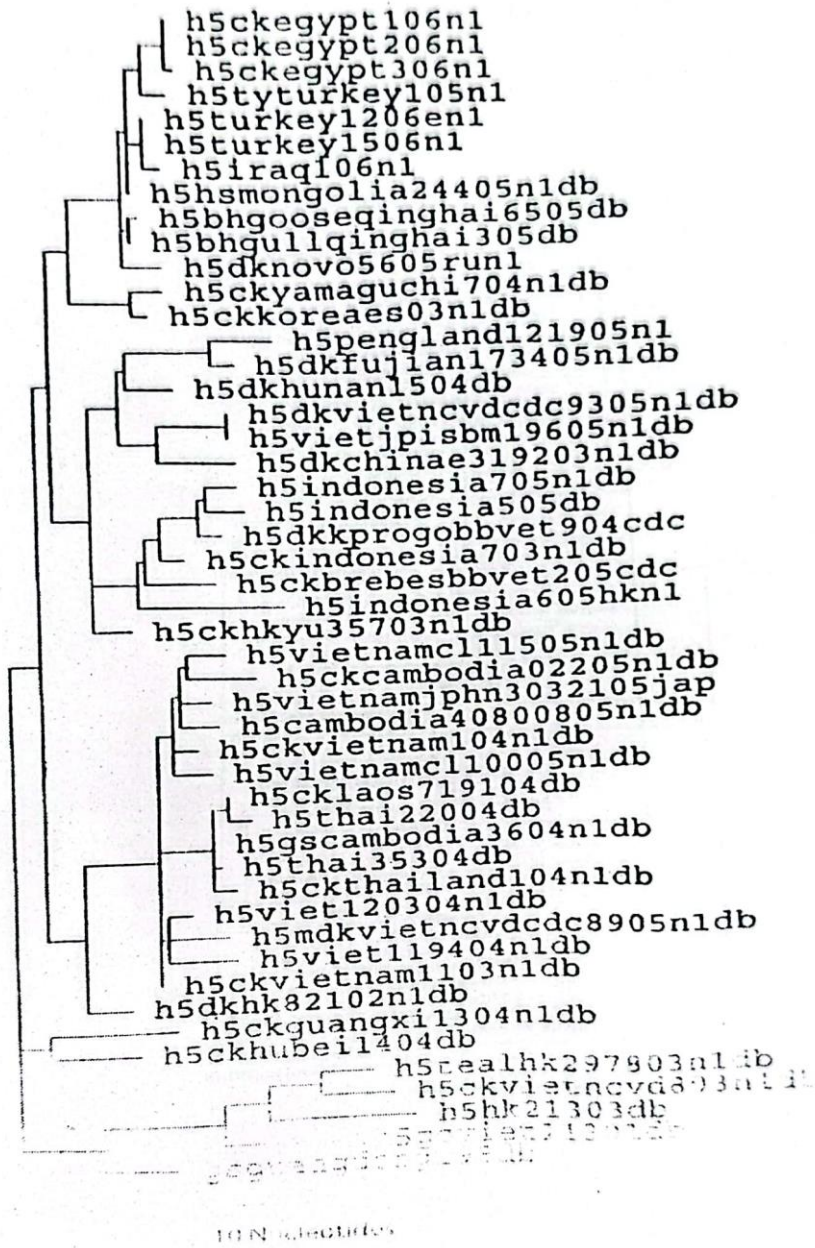


Fig. (2) Phylogenetic comparison of H5 NA genes of outbreaks 2006

Phylogenetic comparison of H5 M genes

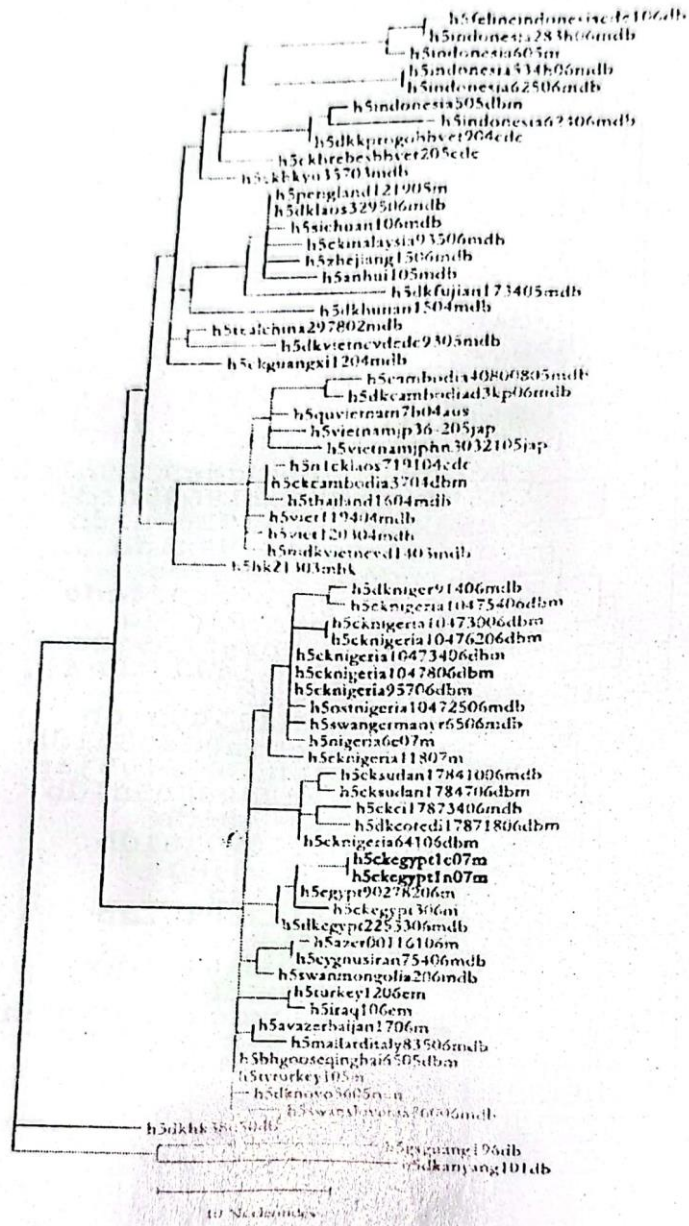


Fig.(3) Phylogenetic comparison of AI H5 M genes of outbreaks 2007

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Phylogenetic comparison of H5 HA genes

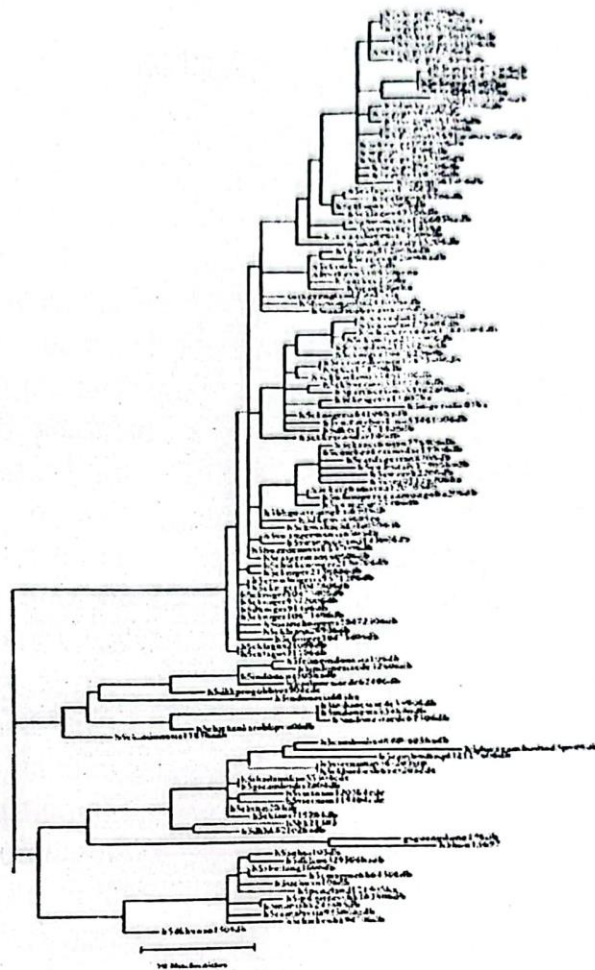


Fig.(4) Phylogenetic comparison of AI H5 HA genes of outbreaks 2007

Phylogenetic comparison of H5 N1 genes

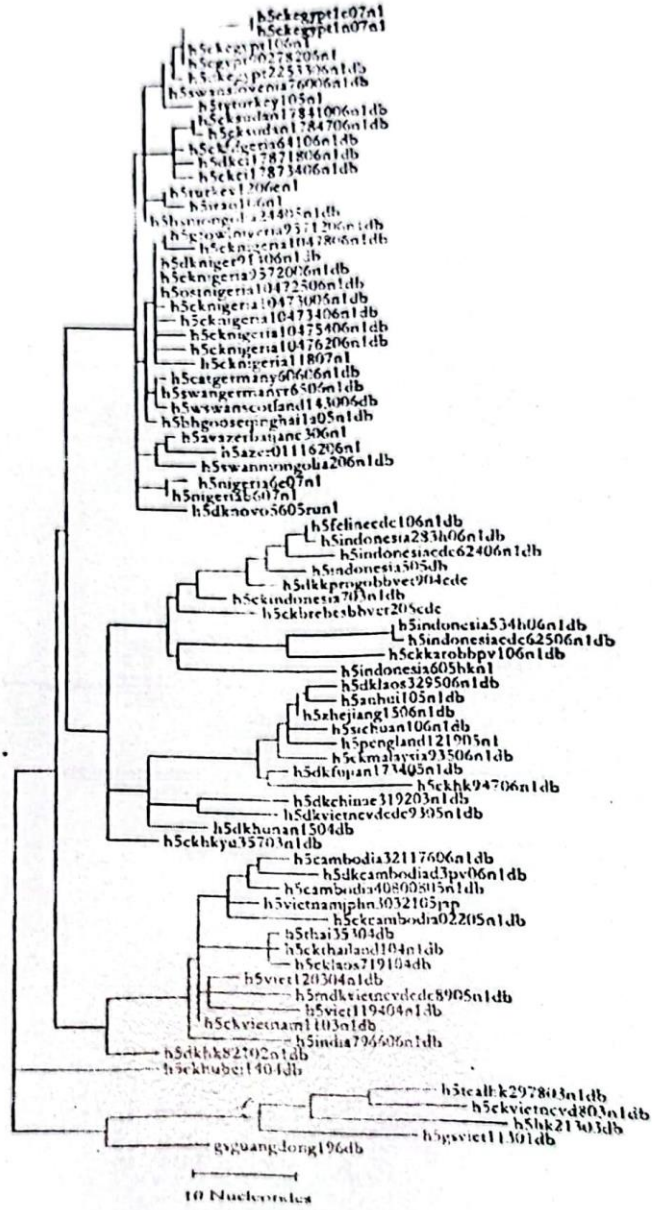


Fig.(5) Phylogenetic comparison of AI H5 N1 genes of outbreaks 2007

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DISCUSSION

Since the HPAI H5N1 virus 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 (16). 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(16).

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(10). It is very important to mentioned that, the wide spread of HPAI since 2003 to about 60 countries allover 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 Gaza 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 (3). 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

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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 (**h5ckeegypt 106 ha** , **h5ckeegypt 206 ha** , **h5ckeegypt 306 ha** / **h5ckeegypt 106 n** , **h5ckeegypt 106 n** , **h5ckeegypt 106 n1**), are closely related to H5N1 strain isolated from turkeys located in Turkey at 2005 (**A/ty/Turkey/1/05**) influenza strain and to H5N1 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 (**h5ckeegypt 1c 07m**, **h5ckeegypt 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).

On the basis of H5 HA genes analysis of Egyptian isolates during 2007 (**h5ckeegypt 1c 07ha**, **h5ckeegypt 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 (5, 6, 12, and 17).

Moreover, the phylogenetic comparison of H5 N1 genes of these isolated strains (**h5ckeegypt 1c 07n1**, **h5ckeegypt 1n 07n1**) revealed the same lineages (Fig. 5) The phylogenetic analysis of Egyptian strains isolated in 2007 (**h5ckeegypt 1c 07m**, **h5ckeegypt 1n 07m** /or **h5ckeegypt 1c 07ha**, **h5ckeegypt 1n 07ha** / or **h5ckeegypt 1c 07n1**, **h5ckeegypt 1n 07n1**) in bold words in fig. 3,4

&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 (8) 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, 2, 3, 4&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, 3 &4)

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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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