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Characterization of H5 Hemagglutinin of H5N1 Avian Influenza Virus in the Middle East

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Abstract: Fifty three of Middle East avian influenza virus H5N1 strains were retrieved from GenBank Database. Other 23 H5N1 representative strains from Asian, Europe, Africa, and North America were also used in this study. Phylogenetic analysis and pairwise comparison of nucleotide and amino acid sequences of.hemagglutinin (HA) gene region of these strains revealed that the H5N1 viruses circulating in Middle East displayed high similarity. The HA protein of the virus contained multiple basic amino acid residues (QGERRRKKR) or (QGEGRRKKR) adjacent to the cleavage site between the HA1 and HA2 domains, showing the highly pathogenic characteristics. Further analyses of these H5N1 strains showed that all of them carried (Gln) 238Q and (Gly) 240G at the receptor binding pocket which indicates preferential binding to alpha-2,3-NeuAcGal receptors. Phylogenetic analysis also confirmed that these H5N1 viruses belonged to the Qinghai lineage or Astrakhan lineage of highly pathogenic avian influenza (HPAI) viruses H5N1 (subclade 2.2 or EMA). As a result, 2 closely related but distinguishable H5N1 sub-subclades are defined and called (2.2.1 (EMA-1) and 2.2.3 (EMA3).

Keywords: Avian influenza virus, H5N1, EMA-1, EMA-3, Middle East.

وصف الجين المخثرH5 لفيروس انفلونزا الطيورH5N1 فى الشرق الاوسط

ملخص: لقد تم استرجاع سلاسل النيوكلتيدات والأحماض الأمينية ل 76 سلالة أنفلونزا الطيور نوع H5N1 من قاعدة البيانات في بنك الجينات. كانت 53 سلالة قد تم عزلها من دول في الشرق الأوسط، أما السلالات الأخرى كانت ممثلة لآسيا ، أوروبا ، أفريقيا ، وأمريكا الشمالية. تحليل تطور السلالات والمقارنة لسلاسل الأحماض الأمينية للجين H5 كشفت أن فيروسات H5N1 المتواجدة في الشرق الأوسط على درجة عالية من التشابه. مقارنة سلاسل الأحماض الأمينية فقد أظهر أن موقع الانشطار يحتوي على العديد من الأحماض الأمينية القاعدية وهذا ما يفسر خاصية الامراضية الشديدة لدى هذه السلالات. كما أظهرت مقارنة سلاسل الأحماض الأمينية أن الجيب لخاص الذي يرتبط مع المستقبل يحتوي على الحامضين الأمينيين جلوتامين (238) وجلايسين(240) مما يشير إلى تفضيلية الارتباط مع المستقبلات ذات العلاقة بفيروسات

أنفلونزا الطيور. تحليل تطور السلالات فقد أظهر أن سلالات**H5N1** المتواجـدة فـي الشـرق الأوسط تنتمي لذرية Qinghai أو Astrakhan واللتان تنتميان لزميرة (EMA أو2,2)، كذلك أثبتت الدراسة أن فيروسات H5N1 المتواجدة في الشرق الأوسط تنتميان إلى تحت زميرتين هما 2.2.1 (EMA-1) و 2.2.3 (EMA-3).

Introduction:

The influenza viruses are medium-sized, comprising enveloped and negative sense RNA viruses with a segmented genome. Taxonomically, they belong to the virus family Orthomyxoviridae. There are three genetically and antigenically distinct types of influenza viruses called A, B, and C. Type A viruses are further divided into subtypes according to the combination of two main envelope glycoproteins the hemagglutinin (HA)and neuraminidase (NA). To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) have been found and almost all subtype combinations (1,2).Avian influenza virus infection can result in huge agricultural and economic consequences, and human morbidity and mortality. Phylogenetic studies have identified nine major clades of H5N1 highly pathogenic avian influenza(HPAI) viruses of Asian origin (3). Clade 2 appears to be most diversified, and members of subclade 2.2 have been shown responsible for the westward spread of H5N1 since 2005 (4). Meanwhile, within subclade 2.2 three further subtypes or (sub-subclades) have been distinguished and were designated with respect to their geographic origin European-Middle East-African (EMA-1–3) (5).

Occasionally the influenza viruses can cross the species barrier and transmit to different mammalian species. The transition of a low pathogenic avian influenza (LPAI).virus to HPAI virus generally results from the introduction of multiple basic amino acids at the HA cleavage site, facilitating systemic virus replication and a mortality of up to100% in poultry. Highly pathogenic avian influenza A virus, H5N1 subtype originating from avian species was the first documented instance from infected humans in Hong Kong in 1997; demonstrating direct transmission of the H5N1 subtype to humans (6). Avian influenza virus H5N1 emerged in 1996 in China and has further expanded its geographical range to affect poultry and humans in Asia, Europe and Africa. As of January 3, 2007, 348 laboratory confirmed human cases of H5N1 infections with 62.1% of these cases have been fatal (216/348) were reported from 14 countries (7).

Characterization of H5 hemagglutinin of H5N1

The current study was conducted to elucidate the phylogenetic relationships between H5N1 isolates in Middle East and to gain further insights into the origin of the virus and to provide clues highlighting putative ways of introduction and also, analysis amino acid sequences of the cleavage site to determine their pathogenecity.

Materials and Methods:

Nucleotide and amino acid sequences of HA gene of seventy six avian influenza virus H5N1 strains were retrieved from GenBank Database by using Basic BLAST engine (www.ch.embnet.org/software/bBLAST.html). Fifty three of these strains were recovered from different Middle East countries, 3 from Palestine, 5 from Israel, 20 from Egypt, 6 from Iraq, 4 from Turkey, 1 from Iran, 2 from Azerbaijan, 5 from Afghanistan and 7 from Sudan. GenBank accession numbers, names, countries and hosts of strains used in this study are listed in Table 1. Phylogenetic analysis was carried out on the HA gene region including nucleotides 93–1689 (1596 nt). The phylogenetic relationship was established by comparison with the representative Asian H5 HA strains (China, Russia, Mongolia, Thailand and Indonesia) and available sequences from Europe (Italy, Germany, Slovenia, Denmark, Austria, Czech Republic, Switzerland, and Scotland), North America (USA and Canada) and Africa (Burkina Faso and Ivory Coast).

Multiple and pairwise sequence alignments were constructed using the clustal algorithm of the computer program CLC free workbench 4.5.1 JRE software (developed by CLC bio A/S, www.clcbio.com). Phylogenetic tree was constructed using the program UPGMA in the same software. The robustness of the groupings in the UPGMA analysis was assessed with 100 bootstrap resamplings. DNA distances for certain strains were created by Kimura 2-parameter Distance method using Phylip programs (8).

Table 1. GenBank accession numbers, names, countries and hosts of H5N1 strains used in this study

No.	Accession number	Strain's name	Country	Host
1	EF532632	A/duck/Gaza/760/2006	Palestine	duck
2	EF532622	A/duck/Gaza/834/2006	Palestine	duck
3	EF532628	A/chicken/Gaza/450/2006	Palestine	chicken
4	EF532627	A/turkey/Israel/446/2006	Israel	turkey
5	EF532629	A/chicken/Israel/625/2006	Israel	chicken

6	EF532625	A/turkey/Israel/365/2006	Israel	turkey
7	EF532623	A/turkey/Israel/345/2006	Israel	turkey
8	EF532626	A/chicken/Israel/397/2006	Israel	chicken
9	DQ862001	A/chicken/Egypt/2253-1/2006	Egypt	Chicken
10	EF441279	A/chicken/Egypt/1081-NAMRU3/2006	Egypt	Chicken
11	EU146866	A/chicken/Egypt/3/2006	Egypt	chicken
12	EU183323	A/chicken/Egypt/F3/2006	Egypt	Chicken
13	EF469653	A/chicken/Egypt/1889N3-SM26/2007	Egypt	Chicken
14	EF042622	A/chicken/Egypt/10845-NAMRU3/2006	Egypt	Chicken
15	EF469651	A/chicken/Egypt/12378N3-CLEVB/2006	Egypt	Chicken
16	EF469650	A/chicken/Egypt/1129N3-HK9/2007	Egypt	Chicken
17	EF042624	A/teal/Egypt/14051-NAMRU3/2005	Egypt	teal
18	CY020653	A/turkey/Egypt/2253-2/2006	Egypt	turkey
19	EU183322	A/turkey/Egypt/F2/2006	Egypt	turkey
20	CY016899	A/duck/Egypt/2253-3/2006	Egypt	duck
21	EF469657	A/duck/Egypt/1888N3-SM25/2007	Egypt	duck
22	EU095024	A/Egypt/2992-NAMRU3/2006	Egypt	Human
23	EU095023	A/Egypt/2991-NAMRU3/2006	Egypt	Human
24	EF042620	A/Egypt/5494-NAMRU3/2006	Egypt	Human
25	EUO95023	A/Egypt/902786/2006	Egypt	Human
26	DQ837589	A/Egypt/2947-NAMRU3/2006	Egypt	Human
27	EF200513	A/Egypt/14725-NAMRU3/2006	Egypt	Human
28	EU146867	A/Egypt/902782/2006	Egypt	Human
29	DQ435200	A/Domestic cat/Iraq/820/2006	Iraq	Domestic cat
30	DQ435201	A/Domestic goose/Iraq/812/2006	Iraq	Domestic goose
31	EU146877	A/Iraq/754/2006	Iraq	Human
32	Iraq/756/2006	A/Iraq/756/2006	Iraq	Human
33	EU146870	A/Iraq/1/2006	Iraq	Human
34	EU146876	A/Iraq/659/2006	Iraq	Human
35	EF619980	A/turkey/Turkey/1/2005	Turkey	turkey

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36	EF619998	A/Turkey/65596/2006	Turkey	Human
37	EF619982	A/Turkey/12/2006	Turkey	Human
38	EF619990	A/Turkey/651242/2006	Turkey	Human
39	CY016779	A/Cygnus cygnus/Iran/754/2006	Iran	Cygnus cygnus
40	EU146873	A/Azerbaijan/011-162/2006	Azerbaijan	Human
41	EU146875	A/Azerbaijan/002-115/2006	Azerbaijan	Human
42	CY020637	A/chicken/Afghanistan/1573-92/2006	Afghanistan	Chicken
43	CY020629	A/chicken/Afghanistan/1573-65/2006	Afghanistan	Chicken
44	CY021373	A/chicken/Afghanistan/1573-7/2006	Afghanistan	Chicken
45	CY020621	A/chicken/Afghanistan/1573-47/2006	Afghanistan	Chicken
46	CY016787	A/chicken/Afghanistan/1207/2006	Afghanistan	Chicken
47	DQ862000	A/chicken/Sudan/2115-9/2006	Sudan	Chicken
48	CY016300	A/chicken/Sudan/1784-10/2006	Sudan	Chicken
49	DQ862003	A/chicken/Sudan/1784/2006	Sudan	Chicken
50	CY021389	A/chicken/Sudan/2115-10/2006	Sudan	Chicken
51	DQ861999	A/chicken/Sudan/2115-12/2006	Sudan	Chicken
52	CY020661	A/chicken/Sudan/1784-8/2006	Sudan	Chicken
53	CY016292	A/chicken/Sudan/1784-7/2006	Sudan	Chiken
54	DQ095619	A/Bar-headed goose/Qinghai/75/05	China	Bar-headed goose
55	DQ997262.	A/swine/Guangxi/wz/2004	China	swine
56	CY016284	A/chicken/Nigeria/957-20/2006	Nigeria	Chicken
57	CY017035	A/Cygnus olor /Italy/742/2006	Italy	Cygnus olor
58	CY020349	A/Cygnus olor/Italy/808/2006	Italy	Cygnus olor
59	CY017043	A/swan/Slovenia/760/2006	Slovenia	swine
60	DQ358746	A/Cygnus olor/Astrakhan/Ast05-2-3/2005	Russia	Cygnus olor
61	EF523692	A/Tufted duck/Denmark/6540/06	Denmark	Tufted duck
62	EF395845	A/swan/Austria/216/2006	Austria	swan

63	DQ515984	A/Cygnus olor/Czech Republic/5170/2006	Czech Republic	Cygnus olor
64	EU016354	A/duck/Switzerland/V487/2006	Switzerland	duck
65	AM492165	A/Stone marten/Germany/R747/2006	Germany	Stone marten
66	EU277833	A/chicken/Burkina Faso/1347-16/2006	Burkina Faso	Chicken
67	CY021517	A/Chicken/Ivory Coast/1787-35/2006	Ivory Coast	Chicken
68	AB233319	A/Bar-headed goose/Mongolia/1/05	Mongolia	Bar-headed goose
69	DQ083565	A/chicken/Saraburi/Thailand/CU-17/04	Thailand	Chicken
70	EF473081	A/chicken/Indonesia/11/2003	Indonesia	Chicken
71	AY059481	A/Duck/HK/2986.1/2000	Hong Kong (China)	duck
72	CY015081	A/chicken/Scotland/1959	Scotland	Chicken
73	CY014726	A/duck/Minnesota/1525/1981	USA	duck
74	EF607855	A/Mute swan/MI/451072-2/2006	USA	Mute swan
75	EF607853	A/Black duck/NC/674-694/2006	USA	Black duck
76	EF405825	A/mallard/ON/499/2005	Canada	mallard

Results:

All Middle East isolates shared features characteristic for recent HPAI virus H5N1 isolates of Asian origin. The alignment of the deduced amino acid sequences of the H5 HA genes showed that isolates in Middle East had the same multiple basic amino acids at the connecting peptide between HA1 and HA2 (QGERRRKKR) except those in Sudan have (QGEGRRKKR), which is a common criterion for the classification of the virus as HPAI. Isolates chicken/Scotland/1959; duck/Minnesota/1525/1981; Mute swan/MI/451072-2/2006; Black duck/NC/674-694/2006 and mallard/ON/499/2005 used here as an outgroup representative, represents an exception as it possesses a cleavage site of LPAIV (Q----RKKR). In addition, all Middle East H5N1 strains included in this study carried HA (Gln) 238Q and HA (Gly) 240G (numbering from the initiating methionine residue) at the receptor binding pocket which indicates preferential binding to alpha-2,3-NeuAcGal receptors, which is typical for influenza viruses of

birds. For the phylogenetic analysis the H5 HA gene of H5N1 viruses isolated in the Middle East revealed that the hemagglutinin molecules of the H5N1 viruses circulating in Middle East during 2006 are highly similar. The genetic homology among Middle East H5N1 isolates were more than 98.42% and had nucleotide sequence identity more than 98.99% with A/Bar-headed goose/Qinghai/75/05 and more than 98.67% with the A/Cygnus olor/Astrakhan/Ast05-2-3/2005 viruses Table2.

Phylogenetic analysis also confirmed that these H5N1 viruses belonged to the Qinghai or Astrakhan lineages of HPAI virus H5N1 (subclade 2.2). Further analyses of the HA gene showed that 2 different clusters are distinguishable among the H5N1 HPAI viruses isolates from Middle East and these are known as EMA-1(2.2.1) and EMA-2 (2.2.3) Figure 1.

Table 2. Percentage of genetic differences between segment H5 sequences derived from 18 H5N1 strains recovered in Middle East and were retrieved from GenBank Database, other 3 representative strains were included for comparison.

29																				
30	0.25																			
33	0.44	0.31																		
37	0.50	0.38	0.44																	
17	0.88	0.76	0.82	0.88																
35	1.01	0.88	0.95	1.01	0.38															
1	1.14	1.01	1.07	1.14	0.50	0.63														
5	1.20	1.07	1.14	1.20	0.57	0.69	0.06													
21	1.33	1.20	1.26	1.33	0.69	0.82	0.31	0.38												
25	1.07	0.95	1.01	1.07	0.44	0.57	0.06	0.13	0.25											
7	1.07	0.95	1.01	1.07	0.44	0.57	0.06	0.13	0.25	0.00										
19	1.14	1.01	1.07	1.14	0.50	0.63	0.13	0.19	0.31	0.06	0.06									
3	1.14	1.01	1.07	1.14	0.50	0.63	0.13	0.19	0.31	0.06	0.06	0.13								
9	1.14	1.01	1.07	1.14	0.50	0.63	0.13	0.19	0.31	0.06	0.06	0.13	0.13							
39	1.07	0.95	1.01	0.95	0.95	1.07	1.20	1.26	1.39	1.13	1.13	1.20	1.20	1.20						
40	1.14	1.01	1.20	1.14	1.14	1.26	1.39	1.45	1.58	1.33	1.33	1.39	1.39	1.39	0.31					
43	1.20	1.07	1.14	1.07	1.07	1.20	1.33	1.39	1.52	1.26	1.26	1.33	1.33	1.33	0.50	0.69				
54	0.69	0.57	0.63	0.57	0.57	0.69	0.82	0.88	1.01	0.76	0.76	0.82	0.82	0.82	0.38	0.57	0.50			
60	1.01	0.88	0.95	1.01	0.88	1.01	1.14	1.20	1.33	1.07	1.07	1.14	1.14	1.14	0.82	1.01	0.95	0.44		
50	1.20	1.07	1.14	1.20	1.07	1.20	1.33	1.39	1.52	1.26	1.26	1.33	1.33	1.33	1.26	1.46	1.33	0.88	1.20	
73	21.48	21.36	21.61	21.20	21.54	21.95	21.61	21.70	22.03	21.70	21.70	21.79	21.61	21.61	21.77	21.61	21.52	21.60	22.10	21.63

Discussion

The first reported isolation of the A/goose/Guangdong/1/96 like lineage viruses, which have multiple basic amino acids at the HA cleavage site and are highly pathogenic for chickens, was obtained from a goose in China in 1996 (9). Then HPAI viruses have caused outbreaks among domestic poultry and wild aquatic birds in many Asian, African, and European countries (5). Influenza A viruses are maintained as biotypes as LPAI viruses in their reservoir hosts, aquatic wild birds. However, upon transmission to gallinaceous poultry viruses of the subtype H5 may mutate to HPAI virus strains by insertional mutation at a sequence of the HA gene encoding an endoproteolytic cleavage site (10). Thus, insertion of basic amino acid residues (arginine, R and lysine, K) can give rise to a polybasic cleavage site accessible to ubiquitous proprotein convertases (11).This molecular characteristic of influenza virus has been identified as a major virulence factor for chickens and turkeys by giving the virus the ability to replicate in a wide range of cell types, resulting in severe disseminated disease and high mortality. The deduced amino acid sequences of H5 HA genes of Middle East strains have a receptor binding pocket of avian origin, which indicate preferential binding to alpha-2,3-NeuAcGal receptors (12). This may explain why an avian virus can be transmitted to humans ineffectively without a change in its receptor binding properties. The difference in host cell receptor specificity may have played a role in restricting transmission of these H5N1 viruses among humans (13).

The HA genes of these isolates although isolated from different hosts, countries and different periods (2005-2007), displayed high homogeneity between nucleotides sequences to gene encoded viral protein responsible for viral attachment. This high similarity between these isolates especially recovered from human and others isolated from avian suggesting the evidence of direct transmission of H5N1 subtype to humans.

Early phylogenetic studies revealed a geographical separation of the avian influenza virus lineage into Eurasian and North American lineages(14). Recently, analysis of 22 complete genomes of H5N1 HPAI virusess isolated from Europe, the Middle East, Russia and Africa. Based on their data three different phylogenetic clades of H5N1 viruses from Europe-Middle East-Africa (EMA) were distinguished (5). Phylogenetically, at least 2 clusters of highly pathogenic H5N1 avian influenza viruses could be distinguished on the basis of their HA gene sequences in Middle East countries. These clusters are similar

EMA-1 and EMA-3 (5) which, according to the most recent nomenclature, should be referred to as sub-subclades 2.2.1 and 2.2.3 (3). Geographic and temporal restrictions of these clusters were evident as 2.2.1 like viruses (EMA-1) were found Egypt, Sudan, Palestine, Israel, Iraq and Turkey, while 2.2.3 like viruses (EMA-3) were found in Afghanistan, Iran and Azerbaijan. The influenza (H5N1) viruses isolated in the Middle East show a close relationship with those isolated in Europe and Africa which related to the same subclade 2.2 (EMA), despite the fact that they were collected from a widely dispersed geographic region, including Sudan, Egypt, Palestine, Israel, Afghanistan, Iran, Azerbaijan, Iraq, Turkey, Nigeria, Ivory Coast, Italy, Czech Republic, Slovenia, Denmark, Switzerland, Germany and Austria. These results are in agreement with that reported previously (5). The shared lineage of the viruses may be explained by a single genetic source for introduction of influenza (H5N1) into Middle East as well as into Europe and Africa;

Our analysis revealed that the hemagglutinin molecules of the H5N1 viruses circulating in Europe, Middle East and Africa during 2005–2007 are highly similar and are apparently the descendants of the Qinghai lineage (China) or Astrakhan lineage (Russia) of HPAI virus H5N1. These results in agreement with data previously reported (3,15-16). Genetic relatedness of H5 HAgene of H5N1 viruses isolated in Middle East as well as in Europe and Africa to those Qinghai lineage or Astrakhan lineage strongly argues that migrating birds can transfer the virus over long distances. Phylogenetic analysis showed that these EMA subclades clearly share a common ancestor in Asia. These results in agreement with data reported recently (5). The 2 sub-subclades may represent separate introductions or, alternatively, a single introduction from Asia into Russia, and Europe or Middle East that has subsequently evolved into 2 lineages. More data will be required to pinpoint when and where these sub-subclades split apart. Phylogenetic tree also showed that sequences of sub-subbclade 2.2.1 (EMA-1) more diversity than sub-subclade 2.2.3 (EMA-3). This might be due to the longer persistence of the infection and, consequently, the chance for an accumulation of mutations or repeated introductions of closely related 2.2.1- like viruses could also have occurred (17).

This study provides a characterization of recent H5N1 viruses isolated from animals and humans in Middle East countries, which has a relationship with the current H5N1 outbreak in Asia. These findings show how DNA analysis of influenza (H5N1) viruses is a method to the better understanding of the

evolution and epidemiology of this virus, which is now present in the 3 continents that contain most of the world's population. This and other related analyses will help us understand the dynamics of infection between wild and domesticated bird populations, which in turn should promote the development of control and prevention strategies. Therefore, further characterization of the H5N1 viruses from different countries is urgently needed.

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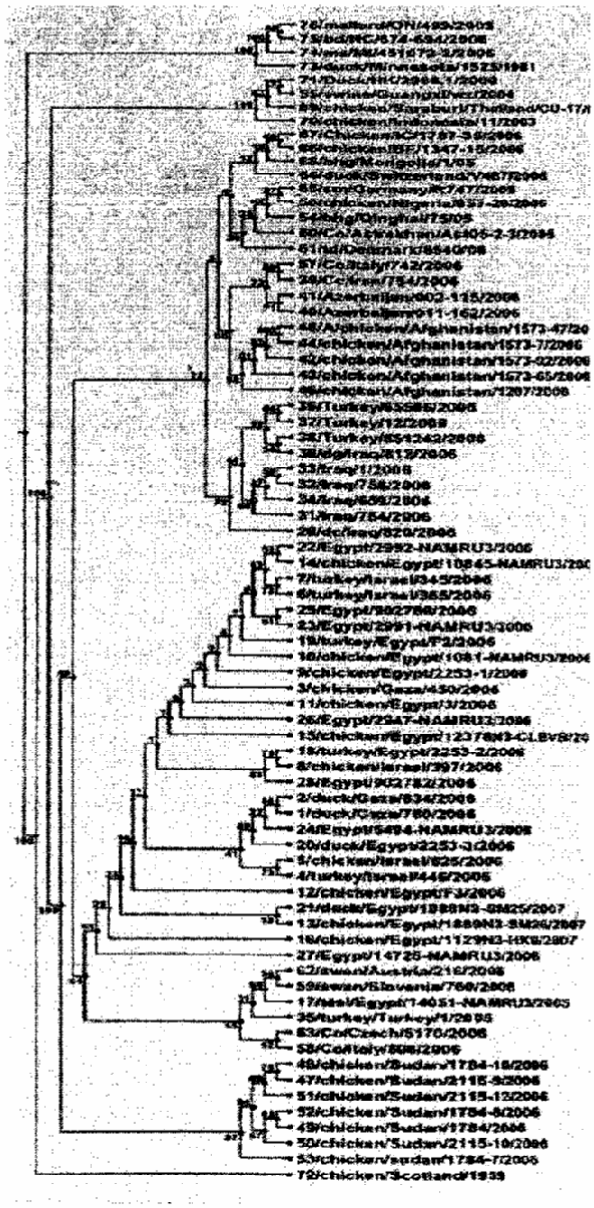

Figure 1. Phylogenetic tree based on HA gene of 53 isolates of H5N1 recovered from different hosts from Middle East countries. Other representative isolates were also included. Analysis was based on nucleotides 93 to 1689 (1596 bp) of HA gene. The robustness of the groupings in the UPGMA analysis was assessed with 100 bootstrap resamplings. H5 HA genes of these strains can be found in the GenBank database. Abbriviation: HK: Hong Kong, IC: Ivory Coast, BF: Burkina Faso, bhg: bar-headed goose, sm: stone marten, Co: Cygnus olor, Cc: Cygnus cygnus, td: tufted duck, dg: domestic goose, dc: domestic cat.