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BRIEF ARTICLE

Evolution of an avian H5N1 influenza A virus escape mutant

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Abstract

AIM: To investigate the genetic constitution of an escape mutant H5N1 strain and to screen the presence of possible amino acid signatures that could differentiate it from other Egyptian H5N1 strains.

METHODS: Phylogenetic, evolutionary patterns and amino acid signatures of the genes of an escape mutant H5N1 influenza A virus isolated in Egypt on 2009 were analyzed using direct sequencing and multi-sequence alignments.

RESULTS: All the genes of the escape mutant H5N1 strain showed a genetic pattern potentially related to Eurasian lineages. Evolution of phylogenetic trees of different viral genes revealed the absence of reassortment in the escape mutant strain while confirming close relatedness to other H5N1 Egyptian strains from human and avian species. A variety of amino acid substitutions were recorded in different proteins compared to the available Egyptian H5N1 strains. The strain displayed amino acid substitutions in different viral alleles similar to other Egyptian H5N1 strains without showing amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

CONCLUSION: The genetic characteristics of avian H5N1 in Egypt revealed evidence of a high possibility of inter-species transmission. No amino acid signatures were found to differentiate the escape mutant H5N1 strain from other Egyptian H5N1 strains.

Key words: Chicken; Genotyping; H5N1; Influenza; Virus evolution

Core tip: The evolution of phylogenetic trees of different viral genes revealed the absence of reassortment in the examined escape mutant H5N1 strain; however, a variety of amino acid substitutions were recorded. The displayed amino acids substitutions in different viral alleles denoted considerable possibility of inter-species transmission, virulence to mammalian species and cytokine resistance.

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INTRODUCTION

The influenza A viruses belong to the Family Orthomyxoviridae. The hemagglutinin (*HA*) and neuraminidase (*NA*) genes encode viral envelope proteins and there are 17 *HA* and 10 *NA* subtypes[1]. Other influenza genes include *PB2*, *PB1*, *PA*, *NS*, *M* and *NP* that encode for viral internal proteins, are required for viral replication and assembly[2] and play an important role in viral infectivity[3]. Reassortments between different influenza A subtypes H9N2 and H5N1 or H7N3 have been detected[4,5]. Interspecies transmission can lead to catastrophic consequences. Egyptian H5N1 viruses are classified as clade

Table 1 Oligonucleotides used for amplification of the *H5N1* genes

Locus	Name	Primer sequence	Length	Amplicon size (bp)	Location	Ref.
N1	N1-F	ATGAATCCAAATCAGAAG	18	1350	21-38	[38]
	N1-R	TGTCAATGGTGAATGGCAAC	20		1346-1365	
PB2	B2-F	GAGGCGATCTGAATTTCG	18	986	1256-1273	[39]
	B2-R	TATGCTAGAGTCCCGTTTCC	20		2222-2241	
PB1	B1F	AGCGAGGAGTATCTGTGAGA	20	601	774-793	[40]
	B1R	TTCCCTCATGATTCGGTGCA	20		1356-1375	
PA	PA-F	ATGAAGAGAGCAGGGCAAGA	20	868	491-510	This study
	PA-R	CAATGGGATACTTCCGCTGT	20		1339-1358	
NP	NP-F	TGCTTGCCTGCTTGTGTGTA	20	665	823-842	[39]
	NP-R	TACTCCTCTGCATTGTCTCCGA	22		1466-1487	
M	M-F	CCCTCAAAGCCGAAATCGCGCA	22	875	56-77	[40]
	M-R	TGCTGTTCCTGCCGATACTCTTCCC	25		906-930	
NS	NS-F	CACTGTGTCAAGCTTTCAGG	20	798	23-42	[39]
	NS-R	TCTCTTGCTCCACTTCAAGC	20		786-805	

2.2.1, which is further subdivided into two groups: A (A1-A5) and B (B1-B2)[6]. The economic consequences, in addition to the zoonotic implications, of highly pathogenic avian influenza virus H5N1 continue to constitute an important problem. According to the recent report of the World Health Organization in June 2013, 628 H5N1 infected cases with 374 fatal consequences were recorded. Egypt is among the countries that contain a very high number of the infected human cases (172) with a total of 62 fatal cases[7]. Endemic situations of H5N1 in Egypt is still an unsolved problem[8]. In Egypt, vaccination of poultry with inactivated vaccine preparations is currently adopted to combat H5N1; however, vaccination of household poultry was suspended in mid 2009 due to the limited impact on H5N1 incidence[8]. In turn, so-called "escape mutants" resulting from antigenic drift of the viruses are selected[9,10]. Escape mutants are known to be less liable to neutralizing antibodies induced by vaccines. Influenza viruses showed a considerable capacity to cross species barriers and to infect and be transmitted among susceptible mammals, including humans. Point mutations and allelic combinations possess a crucial effect on the virulence of HPAI H5N1 isolates and are thought to be polygenic[11,12]. Genetic reassortments among avian influenza viruses are commonly detected in wild bird and poultry isolates[13,14]. The possibility that an avian influenza virus, H5N1, can evolve to human-to-human or mammal-to-mammal transmission through the acquisition of genetic material from the other influenza viruses subtypes already circulating in human or mammals is not weak. The currently studied strain is an escape mutant strain that belongs to 2.2.1, B2 sublineage[10]. The current study aimed to investigate the genetic constitution of the escape mutant strain and compare it with other influenza strains. It also aimed to screen the presence of possible amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

MATERIALS AND METHODS

Viral RNA extraction and RT PCR

Viral RNA was extracted from the infective allantoic fluid of A/chicken/Egypt/F10/2009 using a spin column purification kit (Koma Biotech. Inc., South Korea). Amplification of viral genes was performed with gene-specific primers for *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* (Table 1) using a Koma one step RT PCR kit (Koma Biotech. Inc., South Korea). Following electrophoresis in a 1.5% agarose gel, bands of expected sizes were excised and purified using a QIAquick gel extraction kit (Qiagen, Germany). Purified amplicons were sequenced in both forward and reverse directions (Macrogen, South Korea). Sequences from different genes were routinely assembled and processed. Sequence data of the current study were submitted to the GenBank after removal of trimming primer-linker (Accession No. KC815941-KC815947).

Genetic and phylogenetic analysis

Sequence analysis of the viral genes was conducted using Mega 4.1 as previously described[15]. Sequence alignments of each of the seven genomic segments were conducted using the partial coding regions. Phylogenetic analyses of the A/chicken/Egypt/F10/2009 strain in the current study were conducted with other influenza A viruses to screen the possible reassortant allele. All gene sequence data were collected from the National Center for Biotechnology Information flu database. The neighbor-joining method with Kimura two-parameter distances was used for building the phylogenetic trees using the Mega 4.1[15]. The consistency of the internal branches was evaluated by the p-distance substitution model and 1000 bootstrap replications. The influenza A virus genotype tool at http://www.flugenome.org/genotyping.php[16] was used to determine individual genome segment lineages. A number of human, non-human mammalian and avian viruses were included in the evolutionary trees of *PB2, PB1, PA, NP, NA, M* and *NS* genes with selected sequences from different influenza serotypes in the GenBank to investigate relatedness and possible genetic reassortment.

Deduced amino acid sequence analysis

The multisequence alignment tool available in the flu database was used to compare the deduced amino acid

Table 2 Comparison of amino acid signatures in selected genes of avian and human strains to Egyptian H5N1 strains

Gene	Residue	Avian[1]	Human	Egyptian H5N1	A/CK/Egypt/F10/2009
PB2	475	$L^{214}M^{1}$	$M^{839}L^{3}$	$L^{52}M^{2}$	L
	588	$A^{203}/T^{6}/V^{6}$	$I^{835}/V^{3}/A^{2}$	A^{53}/T^{1}	A
	613	V^{212}/A^{3}	$T^{816}/I^{16}/A^{8}/V^{1}$	V^{54}	V
	627	E^{196}/K^{19}	$K^{838}/R^{2}/E^{1}$	K^{48}/E^{6}	K
	674	$A^{204}/S^{6}/T^{2}/G^{2}/E^{1}$	$T^{836}/A^{2}/I^{2}/P^{1}$	A^{54}	A
PB1	327	R^{147}/K^{3}	K^{766}/R^{66}	R^{58}	R
	336	V^{142}/I^{8}	I^{773}/V^{59}	V^{58}	V
PA	225	$S^{213}C^{1}$	$C^{829}S^{10}$	S	S
	268	L^{214}	$I^{827}K^{11}$	L	L
	356	$K^{212}X^{1}R^{1}$	$R^{827}K^{11}$	K	K
	382	$E^{208}D^{5}V^{1}$	$D^{824}E^{11}V^{2}N^{1}$	E	E
	404	A^{214}	$S^{828}A^{9}P^{1}$	A	A
	409	$S^{189}N^{24}I^{1}$	$N^{830}S^{7}I^{1}$	$S^{77}N^{1}$	S
NP	283	L^{372}/P^{1}	L^{7}/P^{643}	L^{61}	L
	293	R^{371}/K^{2}	R^{28}/K^{622}	R^{60}/K^{1}	R
	305	R^{369}/K^{4}	K^{636}/R^{14}	R^{61}	R
	313	$F^{371}/I^{1}/L^{1}$	Y^{642}/F^{8}	F^{61}	F
	357	$Q^{368}/K^{4}/T^{1}$	$K^{44}/R^{8}/Q^{1}$	Q^{61}	Q
	372	$E^{357}/D^{15}/K^{1}$	D^{630}/E^{23}	E^{61}	E
	422	R^{373}	K^{630}/R^{23}	R^{61}	R
	442	T^{372}/A^{1}	$A^{629}/T^{23}/R^{1}$	T^{61}	T
	455	D^{373}	$E^{630}/D^{22}/T^{1}$	D^{60}/E^{1}	D
M1	115	$V^{856}/I^{2}/L^{1}/G^{1}$	I^{981}/V^{9}	V^{88}	V
	121	$T^{840}/A^{19}/P^{1}$	A^{988}/T^{2}	T^{88}	T
	137	$T^{859}/A^{1}/P^{1}$	A^{974}/T^{12}	T^{88}	T
M2	11	$T^{434}/I^{11}/S^{2}$	I^{911}/T^{44}	T^{90}	T
	20	S^{471}/N^{13}	N^{926}/S^{29}	S^{90}	S
	57	$Y^{481}/C^{1}/H^{1}$	$H^{913}/Y^{33}/R^{2}/Q^{1}$	Y^{90}	Y
NS2	70	$S^{453}/G^{21}/D^{1}$	G^{903}/S^{2}	S^{61}	S

[1]Avian and human amino acid signatures in different viral genes of influenza A viruses as previously determined[20]. Numerical superscripts refer to the number of strains that possess those residues.

Table 3 Amino acid site residues associated with virulence in mammals in comparison with Egyptian isolates

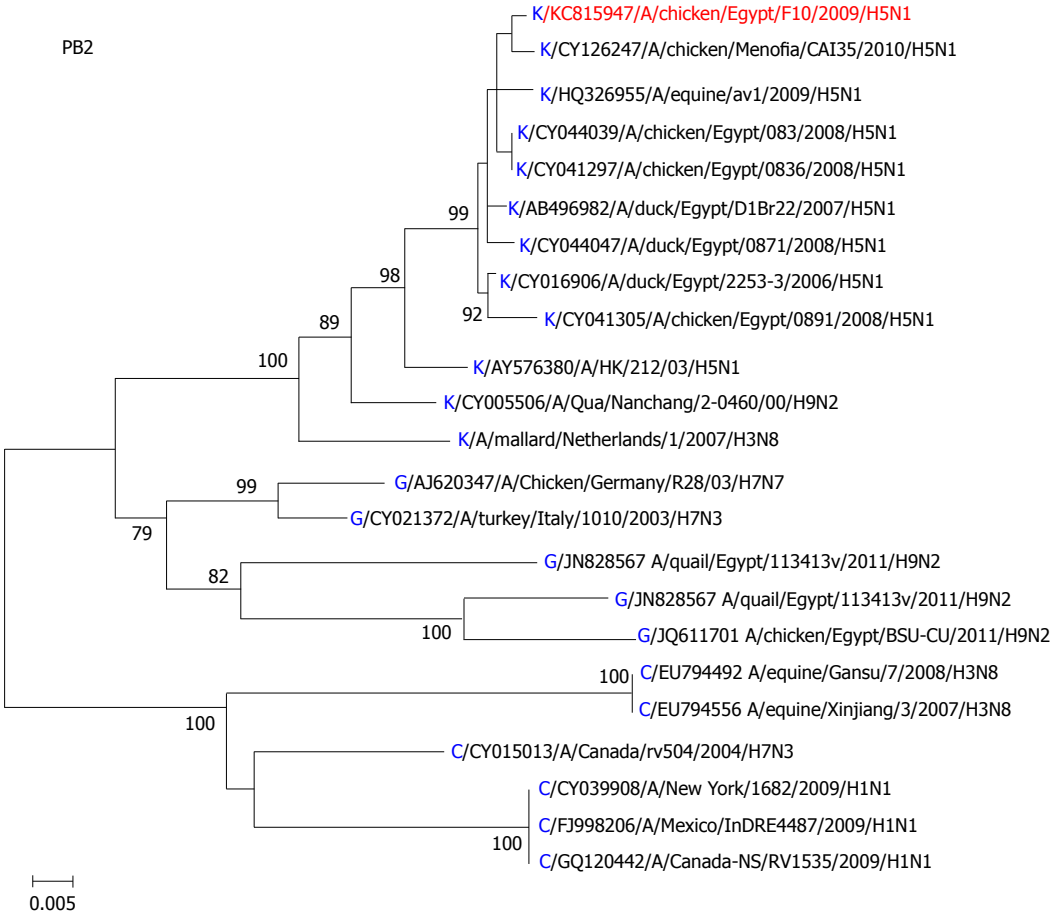
Gene	Site	Residue[1]		Egyptian	A/CK/	Ref.
		Virulent	Avirulent	H5N1 isolates	Eg/F10/09	
PB2	627	K	E	K	K	[11,27]
	701	N	D	D	D	[28]
PB1	317	I	M/V	M/V	M	[11,27]
PA	127	I	V	V	N.I.[2]	[25]
	336	M	L	L	L	[25]
M2	64	S/A/F	P	S	L	[17]
	69	P	L	P	P	[17]
NS1	42	S	A/P	S	S	[29]
	92	E	D	D	D	[27]
	97/92	E	D	E	E	[23]
	127	N	T/D/R/V/A	T/I	I	[30]
	189	N	D/G	D	D	[25]
	195	T/Y	S	S	S	[31]
NS2	31	I	M	M	M	[25]
	56	Y	H/L	H	H	[25]

[1]Virulent and non virulent amino acid residues refer to the ability of the virus to replicate in mammals as determined by Lycett *et al*[17]. [2]N.I.: Not included since it is not flanked by the primers used in the current study.

sequences of the seven genes of the A/chicken/Egypt/F10/2009 strain with other H5N1 strains from the Egyptian H5N1 isolates available in the flu database in order to screen amino acid signature and mutation trend change. Amino acid residues that have associated with mammalian virulence were also screened.

RESULTS

A/chicken/Egypt/F10/2009 in the current study is related to B2 sublineage. Eight amino acid substitutions were found in the F10 strain at the amino acid positions P74S, D 97N, H110R, S123P, R140G, F144Y, N165H and A184E. The different alleles of the F10 isolate were located within subtrees of the majority of the Egyptian strains (Figure 1). The influenza genotyping web tool revealed that the alleles of the F10, *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* alleles, are related to K, G, D, F, 1J, F and 1E genotypes respectively. Analysis of the *NA* gene revealed the presence of the 20-amino acid deletion (data not shown) and the presence of amino acid arginine (R) at position 110. The 228 (N to S) substitution is also present in the F10. The six internal genes (*PB2*, *PB1*, *PA*, *NP*, *M* and *NS*) of A/chicken/Egypt/F10/2009 showed avian like amino acid signatures (Table 2). The polymorphic amino acid residues in different protein sequences of the Egyptian human and avian strains in comparison to the current escape mutant strain were screened and the residues were classified as virulent or nonvirulent (Table 3). Five virulent residues were detected in the avian H5N1 strains in *PB2* (K627), *M2* (S64, P69) and *NS1* (S42, E92/97); however, F10 showed only 4 virulent residues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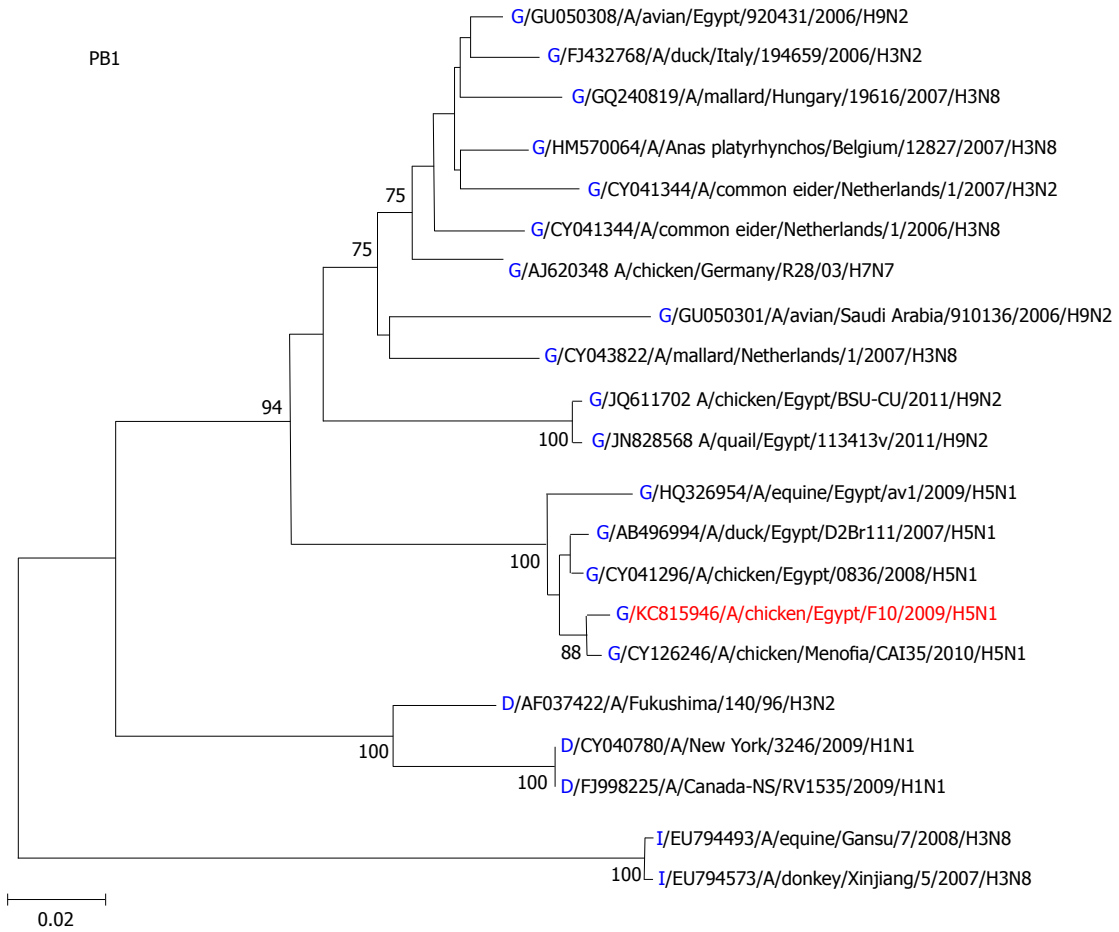
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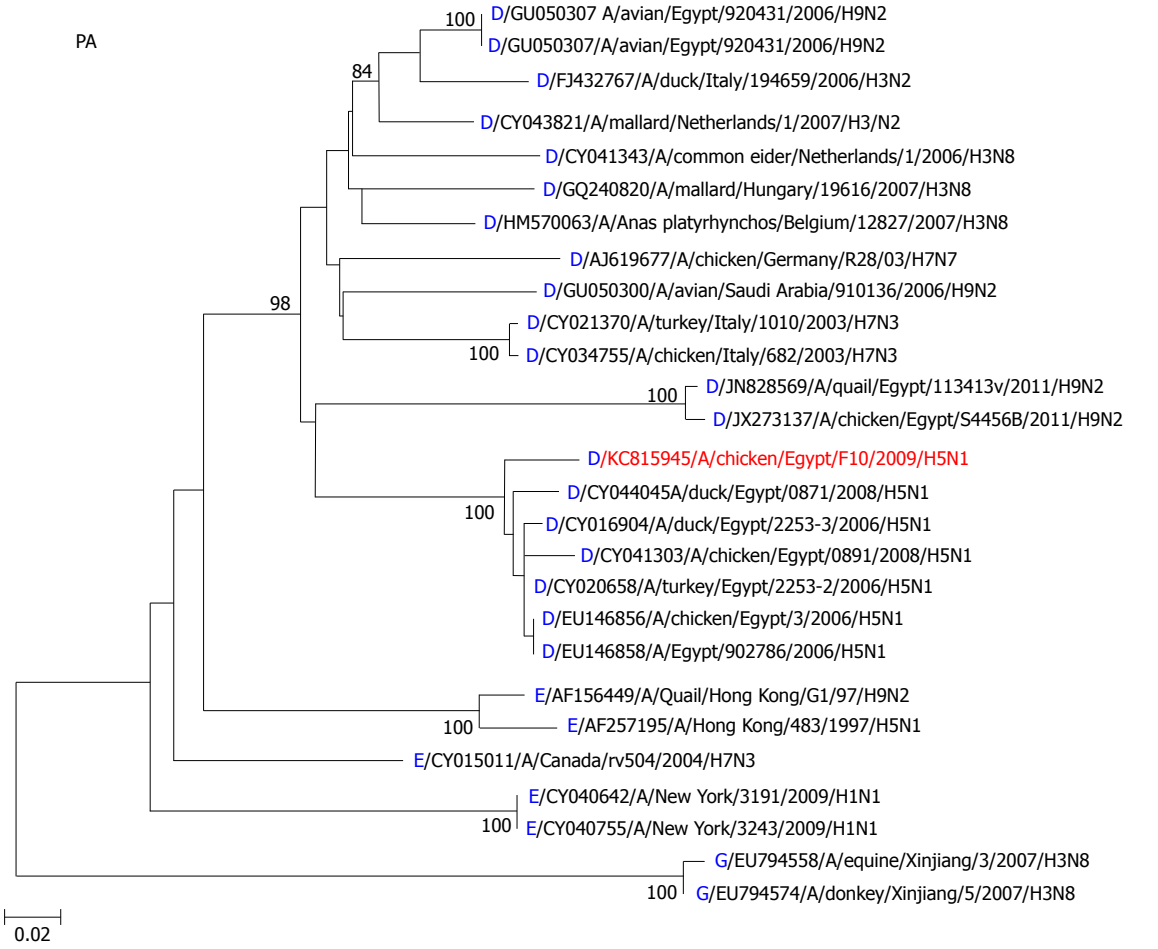
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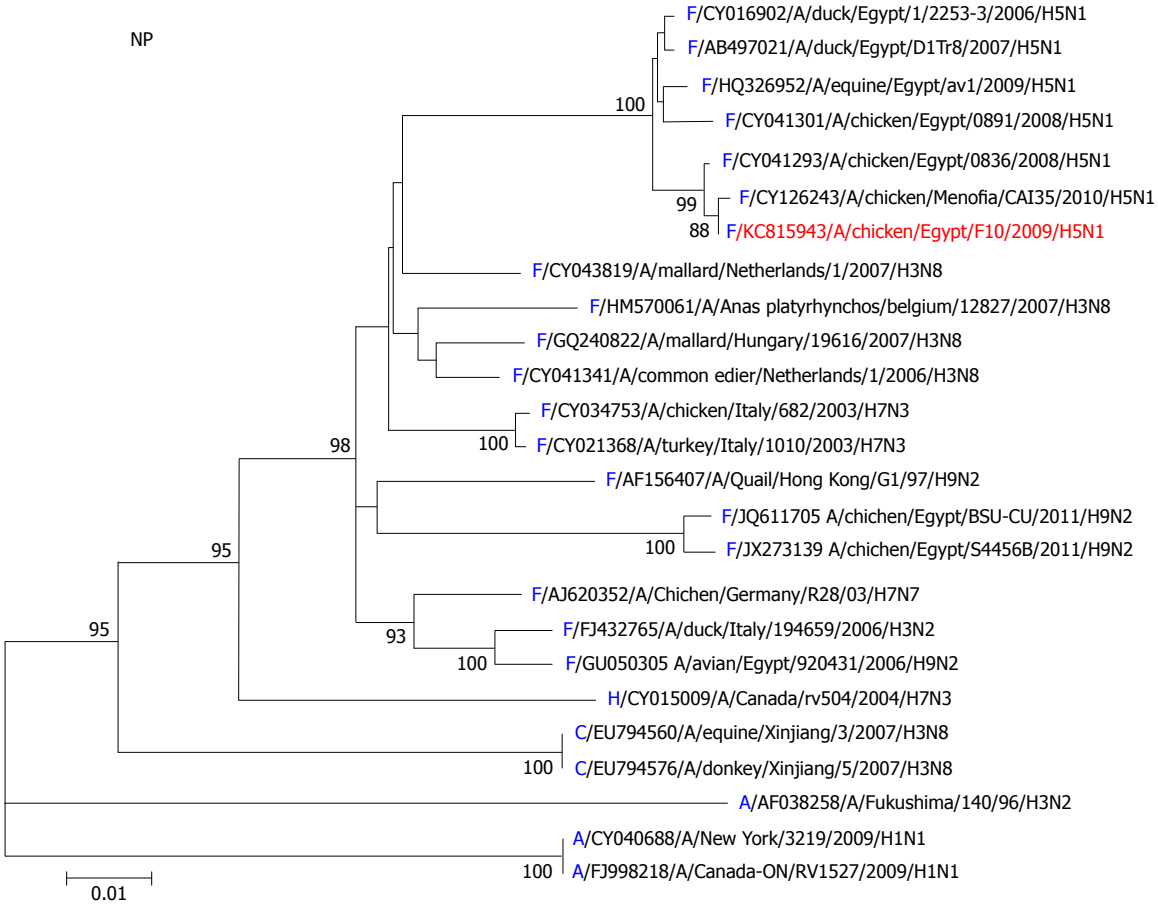
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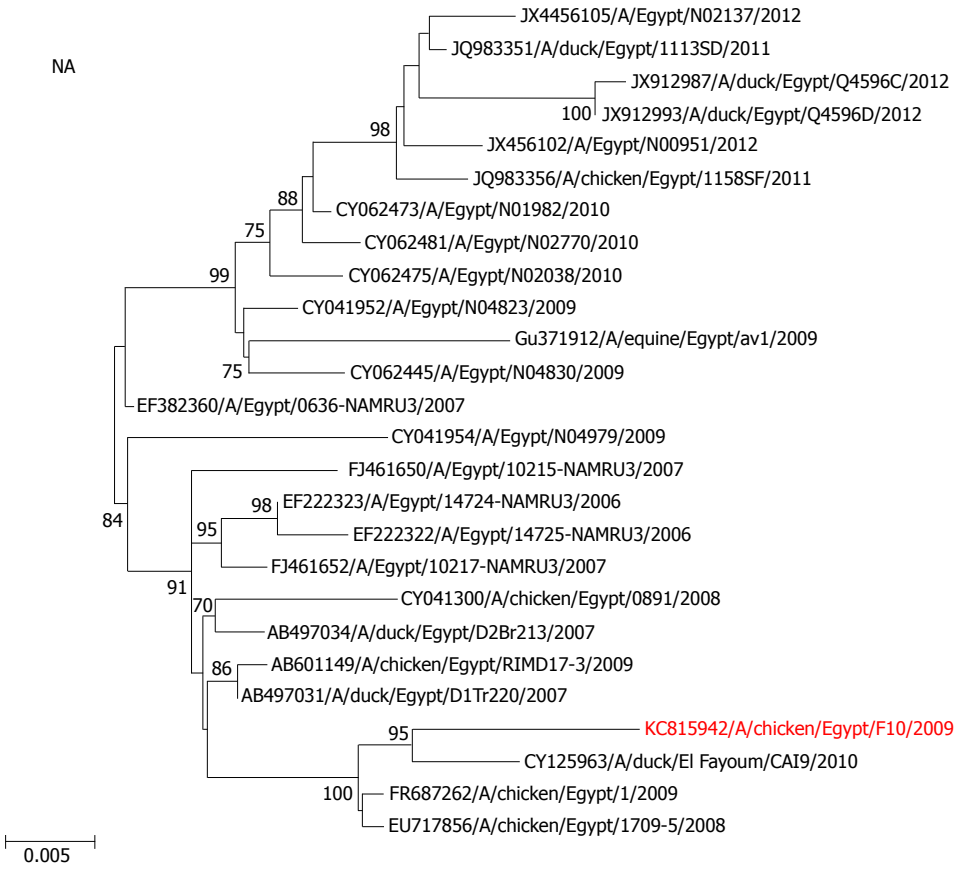
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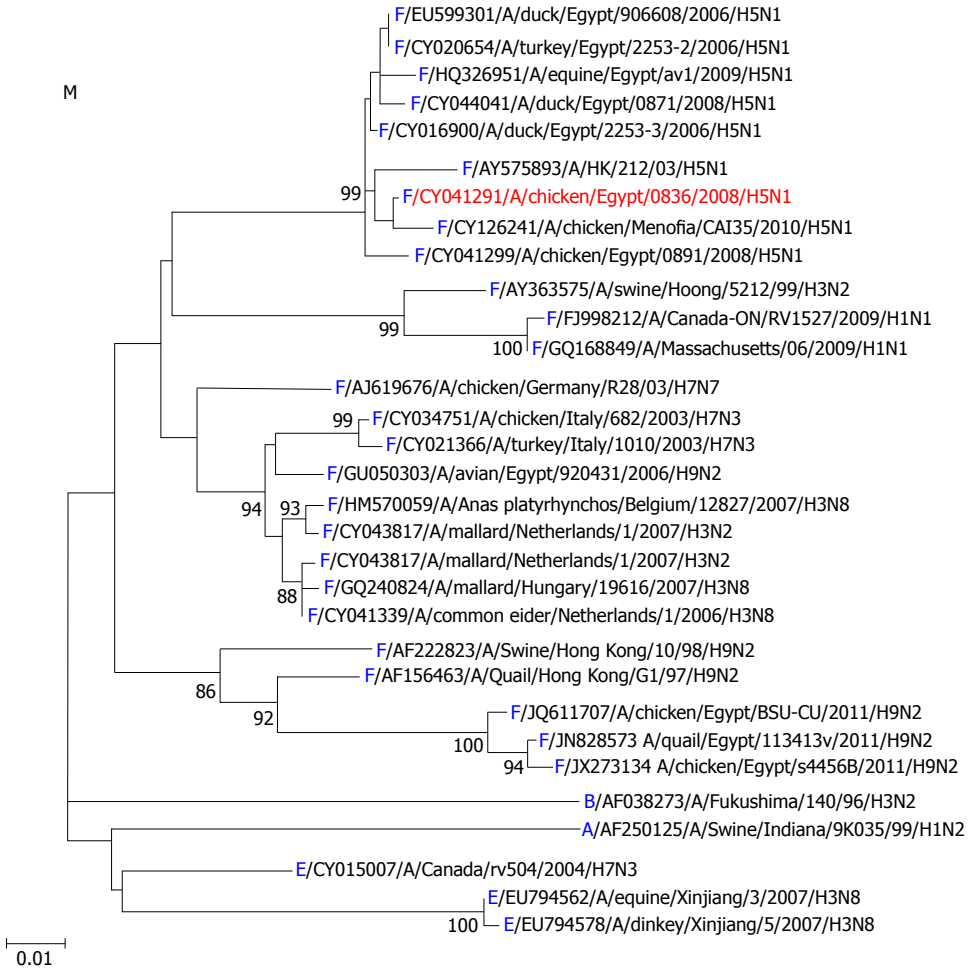
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F/GQ168849/A/Massachusetts/06/2009/H1N1
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F/CY021366/A/turkey/Italy/1010/2003/H7N3
F/GU050303/A/avian/Egypt/920431/2006/H9N2
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F/AF156463/A/Quail/Hong Kong/G1/97/H9N2
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B/AF038273/A/Fukushima/140/96/H3N2
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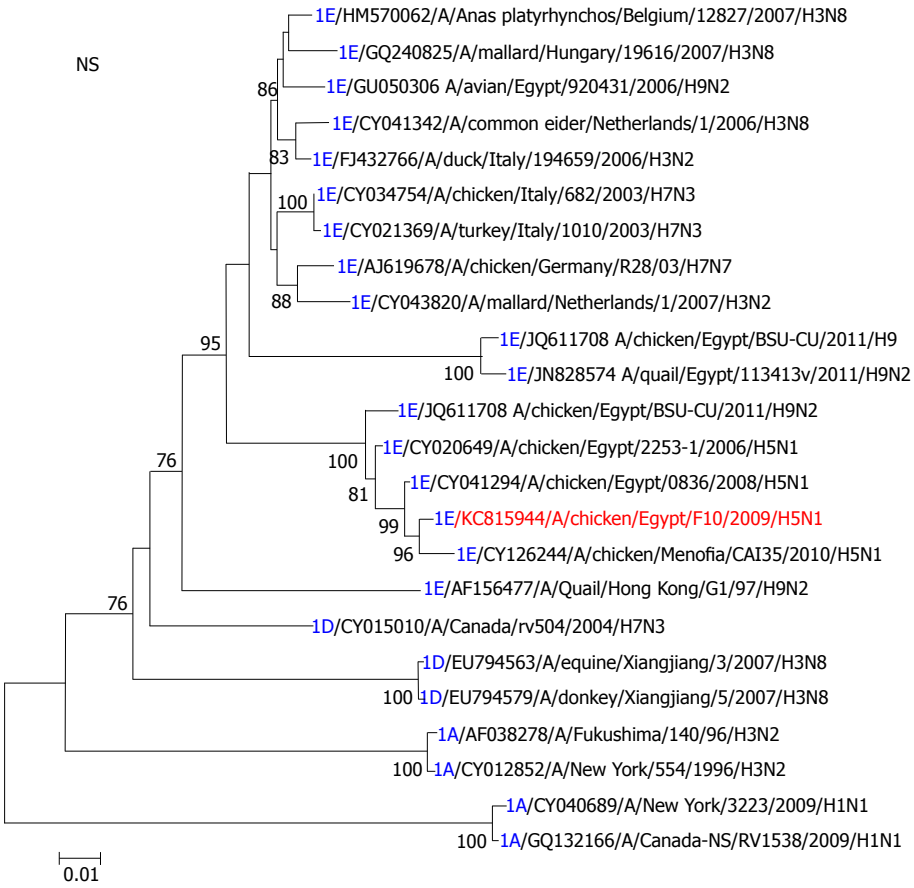

Figure 1 Phylogenetics of the viral genes of escape mutant H5N1 compared to selected influenza viruses. Escape mutant F10 strain examined in the current study was marked by a red color. The selected viruses were chosen to be representative from relevant sequences in GenBank database: H5N1 strains representative to the major gene lineages. Serotypes H1N1, H1N2, H3N2, H3N8, H7N3, H7N7 and H9N2 were also included in the phylogenetic trees of PB2, PB1, PA, NP, M and NS. For NA, Egyptian N1 sequences from Egyptian H5N1 strains were the only included sequences. The robustness of the individual nodes of the tree was assessed using a bootstrap of 1000 resembling in percent (70% and higher). Influenza A virus genotype tool (http://www.flugenome.org/ genotyping.php) was used to determine individual gene segment lineage. The genotype of each strain was mentioned in a blue color in the phylogenetic trees of PB2, PB1, PA, NP, M and NS.

in *PB2* (K627), *M2* (P69) and *NS1* (S42, E92/97). The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion) was observed in this study in the F10 and also in other Egyptian H5N1 strains (Table 3). *PB2* of all Egyptian strains, including avian, mammalian isolates, possessed K627 (Table 3). F10 possessed virulent amino acid substitutions in *PB2* (K627), *M2* (P69) and *NS1* (S42, E97). All the detected virulent residues are also found in the other Egyptian H5N1 strains. Interestingly, all the Egyptian H5N1 strains possess virulent residue S64 in M2 protein while F10 possessed non virulent residue (L64) (Table 3). The *NS1* gene of F10 and other H5N1 Egyptian strains harbored L103F and I106M amino substitutions. The Egyptian H5N1 strains also possessed such amino acid substitutions (data not shown). Egyptian avian H5N1 strains including F10 possessed two human specific residues, E14 and R18 (data not shown).

DISCUSSION

Previous studies revealed that the *HA* genes from H5N1 Egyptian isolates were subjected to cumulative genetic drifts that resulted in further classification of the Egyptian strains into two sublineages [A(A1-A5) and B(B1-B5)][10]. A/chicken/Egypt/F10/2009 in the current study is related to the B2 sublineage. Eight amino acid substitutions were found in the Egyptian variants in lineage B, including the F10 strain at the amino acid positions P74S, D 97N, H110R, S123P, R140G, F144Y, N165H and A184E[10]. The deduced amino acid exchanges, as with most H5N1 Egyptian strains, showed polybasic cleavage motif consensus for clade 2.2 viruses, PGERRRKKR/GLF, while the consensus of 2.2, F10 PQGEGRRKKR/GLF, showed (R325G) substitution[10] with unknown significance. Lycett *et al*[17] specified 6 amino acid residues (86V, 124S, L/N138, T/S156, E/R212, T263) that are linked to the virulence of H5N1 in mammals. T156 and T263 were also present in F10 hemagglutinin[10].

In the current study, the different alleles of the F10 isolate were located within subtrees of the majority of the Egyptian strains. The influenza genotyping web tool revealed that the alleles of the F10, *PB2*, *PB1*, *PA*, *NP*, *NA*, *M* and *NS* alleles, are Eurasian in origin and related to K, G, D, F, 1J, F and 1E genotypes respectively[16].

Analysis of the *NA* gene revealed the presence of

the 20-amino acid deletion, a feature that is frequently observed during the process of adaptation of influenza viruses to poultry that are found to enhance the pathogenesis in chickens. The presence of amino acid arginine (R) at position 110 and the amino acid deletion in the *NA* are characteristic of clade 2.2 viruses[18]. The 228 (N to S) substitution is also present in the F10 and is an indication of 2.2.1 virus. Four *NA* mutations, E119G, H274Y, R292K and N294S, have been reported to confer resistance to *NA* inhibitors[19] but none were detected in the F10 isolate.

Chen *et al*[20] detected amino acid signatures specific to avian and human influenza A viruses. The six internal genes (*PB2*, *PB1*, *PA*, *NP*, *M* and *NS*) of A/chicken/Egypt/F10/2009 and most of Egyptian H5N1 strains showed avian like amino acid signatures.

Identification of the host range-specific amino acids could assume the functional sites that may mediate a host range. In a previous report, the amino acid sequences of the internal proteins in the Hong Kong poultry H5N1 viruses have been compared with those of other avian and human viruses[21]. The polymorphic amino acid residues in different protein sequences of the Egyptian human and avian strains, in comparison to the current escape mutant strain, were screened and the residues were classified as virulent or nonvirulent; such residues have functional significance for virulence in H5N1 to mammals[17]. Five virulent residues were detected in the avian H5N1 strains in *PB2* (K627), *M2* (S64, P69) and *NS1* (S42, E92/97); however, F10 showed only 4 virulent residues in *PB2* (K627), *M2* (P69) and *NS1* (S42, E92/97). An association between glutamic acid (E) at position 92 of the NS1 protein and resistance of H5N1 virus to interferons and TNF-α has been reported[22]. The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion)[23] was observed in this study in the F10 and also in other Egyptian H5N1 strains. However, Seo *et al*[22] 2004 reported that this substitution possesses low impact in the virulence in mammals. E627K substitution in the PB2 protein is one of the genetic indicators for the adaptation and efficient replication in humans[24,25]. The temperature sensitivity of the virus and the efficacy of viral replication depend on the amino acid residue 627 of PB2. Viruses showing K627 displayed higher activity of the polymerase complex during viral replication at a lower temperature in comparison to viruses displaying E627[26]. Efficient virus replication may explain the wide host range of subtype H5N1 strains and their high virulence[26]. The PB2 of all Egyptian strains, including avian and mammalian isolates, possessed K627.

We have compared the amino acid residues associated with H5N1 virulence in mammals[11,17,23,25,27-31] to their corresponding residues in the A/chicken/Egypt/F10/2009. F10 possessed virulent amino acid substitutions in *PB2* (K627), *M2* (P69) and *NS1* (S42, E97). All the detected virulent residues are also found in the other Egyptian H5N1 strains. Interestingly, all the Egyptian H5N1 strains possess virulent residue S64 in the M2 protein, while F10 possessed non virulent residue (L64). P42S and D97E amino acid substitutions in the NS1 are responsible for the virulence of H5N1 in mammalian species and cytokine resistance[22]. In addition, amino acid substitutions L103F and I106M were found to be adaptive genetic determinants for growth and virulence in the NS1 gene of both mammals and avian[32]; F10 and other H5N1 Egyptian strain harbored these amino substitutions. The G184 that was detected in F10 and other H5N1 Egyptian strains contributes to the cleavage and the polyadenylation specificity factor binding and strongly affected the viral virulence[33].

Amantadine resistance is associated with one of the following M2 residues: 26, 27, 30, 31, 34, or 38[34,35]; however, the Egyptian H5N1 strains did possess such amino acid substitutions. Human, swine and avian specific M2 residues were determined[36]. Egyptian avian H5N1 strains, including F10, possessed two human specific residues, E14 and R18[36,37].

The genetic characteristics of the H5N1 virus isolates from chicken in Egypt provided evidence of a high possibility of inter-species transmission. The examined escape mutant H5N1 strain carried no clear amino acid signatures from other Egyptian H5N1 strains.

COMMENTS

Background

Avian influenza viruses showed considerable capacity to cross species barriers to infect susceptible mammals, including humans. Point mutations and reassortment possess a crucial effect on the virulence of HPAI H5N1. Escape mutants resulting from antigenic drift of the viruses were selected under vaccination. The current study aimed to investigate whether the escape mutant strain (A/chicken/Egypt/F10/2009) possesses reassortant genes or amino acid signatures that differentiate it from other classical strains.

Research frontiers

The high error-prone replication of influenza viruses and vaccination pressure unequivocally enhance the robustness of mutation capacity of the influenza viruses. The amino acid signatures of the escape mutant strains have not been addressed. In this study, the authors demonstrate the genetic constitution of the escape mutant strain and the possible amino acid signatures that could differentiate the escape mutant from other Egyptian H5N1.

Innovations and breakthroughs

Recent reports have highlighted critical amino acid substitutions in different alleles of influenza viruses that are associated with virulence to mammals. Amino acid signatures specific to avian and human influenza A viruses were also determined in previous reports. This study reported the presence of different amino acids substitutions in different alleles related to virulence to mammals; however, it failed to find the presence of prominent amino acid signatures in the examined escape mutant strain.

Applications

By understanding the amino acid substitutions in H5N1 escape mutants, its impact on virulence to mammals and how it could be accelerated under vaccination pressure, the avian influenza control procedure method based on vaccination should be reevaluated.

Terminology

Mutation at the *HA* epitope region is among the strategies the influenza virus uses to escape the immune system and represents the most important hindrance to vaccine development. Meanwhile, mutations in the other viral alleles play a crucial role in modulating virus pathogenicity to the original hosts and inter-species transmission to mammalian species, including humans.

Peer review

The authors studied the genetic constitution of the escape mutant H5N1 strain

in comparison with other influenza viral strains. Possible amino acid signatures were explored for identification of the escape mutant from other Egyptian H5N1 and different proteins with amino acid substitutions were also recorded compared to the available Egyptian H5N1 strains. The paper's scientific content is original and of good quality as a research article.

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