Evolution of an avian H5N1 influenza A virus escape mutant

Kamel M A Hassanin, Ahmed S Abdel-Moneim

Kamel M A Hassanin, Biochemistry Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt
Ahmed S Abdel-Moneim, Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt
Ahmed S Abdel-Moneim, Microbiology Department, Virology Division, College of Medicine, Taif University, Al-Taif 21944, Makkah Province, Saudi Arabia

Author contributions: Both authors contributed equally to this work.

Correspondence to: Ahmed S Abdel-Moneim, Professor of Virology, Virology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef 62511, Egypt. asa@bsu.edu.eg
Telephone: +20-82-2327982 Fax: +20-82-2327982
Received: June 29, 2013 Revised: August 21, 2013 Accepted: September 14, 2013 Published online: November 12, 2013

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.

© 2013 Baishideng Publishing Group Co., Limited. All rights reserved.

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.

Hassanin KMA, Abdel-Moneim AS. Evolution of an avian H5N1 influenza A virus escape mutant. World J Virol 2013; 2(4): 160-169 Available from: URL: http://www.wjgnet.com/2220-3249/full/v2/i4/160.htm DOI: http://dx.doi.org/10.5501/wjv.v2.i4.160

INTRODUCTION

The influenza A viruses belong to the Family Orthomyxoviridae. The hemagglutinin (H-A) and neuraminidase (N-A) genes encode viral envelope proteins and there are 17 H-A and 10 N-A subtypes[6]. Other influenza genes include PB2, PB1, P-A, N3, M and NP that encode for viral internal proteins, are required for viral replication and assembly[7] and play an important role in viral infectivity[8]. Reassortments between different influenza A subtypes H9N2 and H5N1 or H7N3 have been detected[9]. Interspecies transmission can lead to catastrophic consequences. Egyptian H5N1 viruses are classified as clade
2.2.1, which is further subdivided into two groups: A (A1-A5) and B (B1-B2)\[44\]. 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\[5\]. 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\[9\]. 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 H51 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\[14\]. 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 genespecific 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).

### 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  |       | TGGCAATGTCAACGCGAC              | 20     | 1346-1365         |          |        |
| B1-F  |       | CACGTGTCAAGCTTTCAGG             | 18     | 986               | 1256-1273| [39]   |
| PB2   | B1-F  | AGCGAGGATATCTTGAGA              | 20     | 961               | 774-793  | [40]   |
| NP    | N1-F  | TATCTTGGTGTACCTTCTTCT          | 20     | 665               | 1339-1358|        |
| PA    | N1-R  | AACGAGGATATCTTGAGA              | 20     | 639               | 1339-1358|        |
| M     | N2-F  | TGTCAATGGTGAATGGCAAC            | 20     | 592               | 1466-1487|        |
| NS    | N5-F  | ATGCGAGGATATCTTGAGA             | 20     | 464               | 1195-1213|        |
|       | N5-R  | ATGCGAGGATATCTTGAGA             | 20     | 464               | 1195-1213|        |

**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 genotyping 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† | Human | Egyptian H5N1 | A/CK/Egypt/F10/2009 |
|-------|---------|--------|-------|---------------|---------------------|
| PB2   | 475     | L®M®  | M®L®  | L®M®          | L®                 |
|       | 588     | A®T®/†/V® | T®/V®/A® | A®T®          | A®                |
|       | 613     | V®/A® |     |               | V®                 |
|       | 627     | E®/K® | K®/E® |             | K®                |
|       | 674     | A®°/T°/G°/C°/E° | T°/A°/T°/P° | A®          | A®                |
| PB1   | 327     | R®/K® | K®/R® |             | R®                |
|       | 336     | V®/I® | I®/V® |             | V®                |
| PB2   | 225     | S®/R® | C®/S® |             | S®                |
|       | 268     | L®    |     |             | L®                |
|       | 356     | K®/R® | R®/K® |             | K®                |
|       | 382     | E®/D® | D®/E® |             | E®                |
|       | 404     | A®®   |     |             | A®                |
|       | 409     | S®/N® | S®/N® |             | S®                |
| NP    | 283     | L®/I® | L®/I® |             | L®                |
|       | 293     | R®/K® | R®/K® |             | R®                |
|       | 305     | R®/K® | R®/K® |             | R®                |
|       | 313     | F®/I/L® | F®/I/L® |             | F®                |
|       | 357     | Q®/S®/K®/T® | Q®/S®/K®/T® | Q®          | Q®                |
|       | 372     | E®/D®/K® | E®/D®/K® | E®          | E®                |
|       | 422     | R®®   |     |             | R®®               |
|       | 442     | T®/V® | T®/V® |             | T®                |
| M1    | 115     | V®/I/L/G® | V®/I/L/G® | V®          | V®                |
|       | 121     | T®/A®/P® | T®/A®/P® | T®          | T®                |
|       | 137     | T®/A®/P® | T®/A®/P® | T®          | T®                |
| M2    | 11      | T®/I/S® | T®/I/S® |             | T®                |
|       | 20      | S®/N® | S®/N® |             | S®                |
|       | 57      | Y®/S®/H® | Y®/S®/H® | Y®          | Y®                |
| NS2   | 70      | S®/G®/D® | S®/G®/D® | S®          | S®                |

†Avian and human amino acid signatures in different viral genes of influenza A viruses as previously determined[24]. Numerical superscripts refer to the number of strains that possess these residues.

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

| Gene  | Site | Residue† | Virulent | Avirulent | Egyptian H5N1 Isolates | A/CK/Egypt/F10/09 | Ref. |
|-------|------|----------|----------|-----------|------------------------|------------------|------|
| PB2   | 627  | K        | E        | K         | K                      | K                | [11,27] |
|       | 701  | N        | D        | D         | D                      | D                | [28]  |
| PB1   | 317  | I        | M/V      | M/V       | M                      | M                | [11,27] |
|       | 127  | I        | V        | V         | V/L                   | V/L              | [25]  |
| PA    | 336  | M        | L        | L         | L                      | L                | [25]  |
| M2    | 64   | S/A/F/P  | P        | S         | L                      | L                | [17]  |
|       | 69   | P        | L        | P         | P                      | P                | [17]  |
| NS1   | 42   | S        | A/P      | S         | S                      | S                | [29]  |
|       | 92   | E        | D        | D         | D                      | D                | [27]  |
|       | 367  | E        | D        | E         | E                      | E                | [23]  |
|       | 127  | N        | T/D/R/V/A | T/I  | I                      | I                | [30]  |
|       | 189  | N        | D/G      | D         | D                      | D                | [25]  |
|       | 195  | T/Y      | S         | S         | S                      | S                | [31]  |
| NS2   | 31   | I        | M        | M         | M                      | M                | [25]  |
|       | 56   | H        | Y/L/H    | H         | H                      | H                | [25]  |

†Virulent and non-virulent amino acid residues refer to the ability of the virus to replicate in mammals as determined by Lyzette et al.[21]. N.L.: Not included since it is not flanked by the primers used in the current study.

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 subtypes 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, N, M and NS alleles, are related to K, G, D, F, I1, F and I1E genotypes respectively. Analysis of the N/A 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 NS2 (S42, E92/97); however, F10 showed only 4 virulent residues.
Hassanin KMA et al. H5N1 evolution in Egypt
Hassanin KMA et al. H5N1 evolution in Egypt

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 avian 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, PGERRR/KKR/GLF, while the consensus of 2.2, F10 PQGEGRRK/KR/GLF, showed (R325G) substitution with unknown significance. Lycett et al\(^{[7]}\) 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\(^{[9]}\).

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, N, M and NS alleles, are Eurasian in origin and related to K, G, D, F, IJ, F and 1E genotypes respectively\(^{[10]}\).

Analysis of the NA gene revealed the presence of

---

**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, 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.
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 N/A are characteristic of clade 2.2 viruses\cite{18}. The 228 (N to S) substitution is also present in the F10 and is an indication of 2.2.1 virus. Four N/A mutations, E119G, H274Y, R292K and N294S, have been reported to confer resistance to N/A inhibitors\cite{19} but none were detected in the F10 isolate.

Chen et al\cite{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\cite{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\cite{22}. Five virulent residues were detected in the avian H5N1 strains in PB2 (K627), M2 (S64, P69) and N31 (S42, E92/97); however, F10 showed only 4 virulent residues in PB2 (K627), M2 (P69) and N31 (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\cite{23}. The mutation of aspartic acid (D) to glutamic acid (E) at position 92 (97 in strains with 5 amino acids deletion)\cite{24} was observed in this study in the F10 and also in other Egyptian H5N1 strains. However, Seo et al\cite{25} 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\cite{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\cite{26}. Efficient virus replication may explain the wide host range of subtype H5N1 strains and their high virulence\cite{27}. 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\cite{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 N31 (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\cite{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\cite{22}, 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\cite{23}.

Amantadine resistance is associated with one of the following M2 residues: 26, 27, 30, 31, 34, or 38\cite{28,29}; however, the Egyptian H5N1 strains did possess such amino acid substitutions. Human, swine and avian specific M2 residues were determined\cite{30}. Egyptian avian H5N1 strains, including F10, possessed two human specific residues, E14 and R18\cite{30,31}.

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 acid 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...
Hassanin KMA et al. H5N1 evolution in Egypt

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.

REFERENCES

1. Tong S, Li Y, Rivailier P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turnelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci USA 2012; 109: 4269-4274 [PMID: 22371588 DOI: 10.1073/pnas.111620109]

2. Snyder MH, Buckler-White AJ, London WT, Tierney EL, Murphy BR. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. J Virol 1987; 61: 2857-2863 [PMID: 2441080]

3. Guan Y, Peiris JS, Lipatov AS, Ellis TM, Dyerting KC, Krauss S, Zhang LJ, Webster RG, Shortridge KF. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc Natl Acad Sci USA 2002; 99: 8950-8955 [PMID: 12077307 DOI: 10.1073/pnas.132268999]

4. Guan Y, Shortridge KF, Krauss S, Chin PS, Dyerting KC, Ellis TM, Webster RG, Peiris M. H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. J Virol 2000; 74: 9372-9380 [PMID: 11000205 DOI: 10.1128/JVI.74.20.9372-9380.2000]

5. Iqbal M, Yasubq T, Reddy K, McAuley JW. Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PLoS One 2009; 4: e5788 [PMID: 19517011 DOI: 10.1371/journal.pone.0005788]

6. Abdel-Moneim AS, Shany SA, Fereidouni SR, Eid BT, El-Kady MF, Starick E, Harder T, Keil GM. Sequence diversity of the haemagglutinin open reading frame of recent highly pathogenic avian influenza H5N1 isolates from Egypt. Arch Virol 2009; 154: 1559-1562 [PMID: 19669616]

7. Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO. World Health Organization; 2013 Jun 4. Available from: URL: http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/

8. Emergency Prevention System, Food and Agriculture Organization of the United Nations-Global early Warning System. 2010. H5N1 HPAI global overview, vol 3: p 1-8. Food and Agriculture Organization of the United Nations, Rome, Italy

9. Swayne DE, Kapczynski D. Strategies and challenges for avian influenza (H5N1) viruses isolated from humans and poultry in 2004. J Virol 2005; 79: 2191-2198 [PMID: 15681421 DOI: 10.1128/JVI.79.4.2191-2198.2005]

10. Dugan VG, Chen R, Spiro DJ, Sengamalay N, Zaborsky J, Ghedin E, Nolting J, Swayne DE, Runstadler JA, Happ GM, Senne DA, Wang R, Simonds RD, Holmes EC, Taubenberger JK. The evolutionary genetics and emergence of avian influenza viruses in wild birds. PLoS Pathog 2008; 4: e1000076 [PMID: 18561303 DOI: 10.1371/journal.ppat.1000076]

11. Macken CA, Webby RJ, Bruno WJ. Genotype turnover by reassortment of replication complex genes from avian influenza A virus. J Gen Virol 2006; 87: 2803-2815 [PMID: 16963738 DOI: 10.1099/vir.0.81454-0]

12. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001; 17: 1244-1245 [PMID: 11751241 DOI: 10.1093/bioinformatics/17.12.1244]

13. Lu G, Rowley T, Garten R, Donis RO. FluGenome: a web tool for genotyping influenza A virus. Nucleic Acids Res 2007; 35: W275-W279 [PMID: 17537820 DOI: 10.1093/nar/gkm365]

14. Lyczet SJ, Ward MJ, Lewis FL, Poon AF, Kosakovskys Pond SL, Brown A. Detection of mammalian virulence determinants in highly pathogenic avian influenza H5N1 viruses: multivariate analysis of published data. J Virol 2009; 83: 9901-9910 [PMID: 19625397 DOI: 10.1128/JVI.00608-09]

15. Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, Guan Y. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci USA 2006; 103: 2845-2850 [PMID: 16479351 DOI: 10.1073/pnas.0511120103]

16. Chen GW, Chang SC, Mok CK, Li YL, Kung YN, Huang JH, Shih YH, Wang JY, Chiang C, Chen CJ, Shih SR. Genomic signatures of human versus avian influenza A viruses. Emerg Infect Dis 2006; 12: 1353-1360 [PMID: 17073083 DOI: 10.3201/eid1209.060276]

17. Zhou NN, Shortridge KF, Claas EC, Krauss SL, Webster RG. Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. J Virol 1999; 73: 3366-3374 [PMID: 10074191]

18. Seo SH, Hoffmann E, Webster RG. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. Virus Res 2004; 103: 107-113 [PMID: 15163498 DOI: 10.1016/j.virusres.2004.02.022]

19. Long JX, Peng DX, Liu YL, Wu YT, Liu XF. Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes 2008; 36: 471-478 [PMID: 18317917 DOI: 10.1007/s11262-007-0187-8]

20. Shinya K, Hammad S, Hatta M, Ito H, Ito T, Kawakoa Y, PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A virus in mice. Virology 2004; 320: 258-266 [PMID: 15016548 DOI: 10.1016/j.virol.2004.02.007]

21. Subbarao K, Shaw MW. Molecular aspects of avian influenza (H5N1) viruses isolated from humans. Rev Med Virol 2000; 10: 337-348 [PMID: 11015744 DOI: 10.1002/1099-1654(200009)10:5<337::AID-RMV292>3.0.CO;2-V]

22. Massin P, van der Werf S, Naifakh N. Resistance of 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol 2001; 75: 5398-5404 [PMID: 11333924 DOI: 10.1128/JVI.75.13.5398-5404.2001]

23. Lee MS, Deng MC, Lin YJ, Chang CY, Shieh HK, Shiuaj JZ, Huang CC. Characterization of an H5N1 avian influenza virus from Taiwan. Vet Microbiol 2007; 124: 193-201 [PMID: 17512143 DOI: 10.1016/j.vetmic.2007.04.021]

168 November 12, 2013 | Volume 2 | Issue 4 |
28 **Steel J**, Lowen AC, Mubareka S, Palese P. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. *PLoS Pathog* 2009; 5: e1000252 [PMID: 19119420 DOI: 10.1371/journal.ppat.1000252]

29 **Jiao P**, Tian G, Li Y, Deng G, Jiang Y, Liu C, Liu W, Bu Z, Kawaoaka Y, Chen H. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. *J Virol* 2008; 82: 1146-1154 [PMID: 18032512 DOI: 10.1128/JVI.01698-07]

30 **Min JY**, Li S, Sen GC, Krug RM. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. *Virology* 2007; 363: 236-243 [PMID: 17320139]

31 **Bornholdt ZA**, Prasad BV. X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus. *Nature* 2008; 456: 985-988 [PMID: 18987632 DOI: 10.1038/nature07444]

32 **Dankar SK**, Wang S, Ping J, Forbes NE, Keleta L, Li Y, Brown EG. Influenza A virus NS1 gene mutations F103L and M106I increase replication and virulence. *Virology* 2011; 8: 13 [PMID: 21226922 DOI: 10.1186/1743-422X-8-13]

33 **Steidle S**, Martínez-Sobrido L, Mordstein M, Lienenklaus S, García-Sastre A, Stäheli P, Kochs G. Glycine 184 in nonstructural protein NS1 determines the virulence of influenza A virus strain PR8 without affecting the host interferon response. *J Virol* 2010; 84: 12761-12770 [PMID: 20926573 DOI: 10.1128/JVI.00701-10]

34 **Belshe RB**, Smith MH, Hall CB, Betts R, Hay AJ. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. *J Virol* 1988; 62: 1508-1512 [PMID: 3262579]

35 **Schnell JR**, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 2008; 451: 591-595 [PMID: 18235503 DOI: 10.1038/nature06531]

36 **De Filette M**, Fiers W, Martens W, Birkett A, Ramme A, Löwenadler B, Lycke N, Jou WM, Saelens X. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine* 2006; 24: 6597-6601 [PMID: 16814430 DOI: 10.1016/j.vaccine.2006.05.082]

37 **Betakova T**. M2 protein-a proton channel of influenza A virus. *Curr Pharm Des* 2007; 13: 3231-3235 [PMID: 18045172 DOI: 10.2174/138161207782341295]

38 **Abdel-Moneim AS**, Abdel-Ghany AE, Shany SA. Isolation and characterization of highly pathogenic avian influenza virus subtype H5N1 from donkeys. *J Biomed Sci* 2010; 17: 25 [PMID: 20398268 DOI: 10.1186/1423-0127-17-25]

39 **Abdel-Moneim AS**, Shehab GM, Abu-Elsaad AA. Molecular evolution of the six internal genes of H5N1 equine influenza A virus. *Arch Virol* 2011; 156: 1257-1262 [PMID: 21431346 DOI: 10.1007/s00705-011-0966-3]

40 **Abdel-Moneim AS**, Afifi MA, El-Kady MF. Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. *Virol J* 2012; 9: 173 [PMID: 22925485 DOI: 10.1186/1743-422X-9-173]

P- Reviewers: Borgmann S, John JM, Liu XY  S- Editor: Ma YJ  L- Editor: Roemmele A  E- Editor: Wang CH