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ORIGINAL ARTICLE

Is avian influenza A (H7N9) virus staggering its way to humans?

Guang-Wu Chen [a,b,*], Michael M.C. Lai [c,d], Suh-Chin Wu [e], Shih-Cheng Chang [b], Li-Min Huang [f], Shin-Ru Shih [b,g,h,*]

[a] *Department of Computer Science and Information Engineering, College of Engineering, Chang Gung University, Taoyuan, Taiwan*
[b] *Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[c] *China Medical University Hospital, Taichung, Taiwan*
[d] *Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan*
[e] *Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan*
[f] *Department of Pediatrics, National Taiwan University Hospital, Taipei, Taiwan*
[g] *Department of Medical Biotechnology, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[h] *Clinical Virology Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan*

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Background/Purpose: Human infections by a new avian influenza A (H7N9) virus have been reported. As of April 23, 2013, there were 108 confirmed cases including 22 deaths in China.
Methods: Influenza protein sequences were downloaded from the Influenza Virus Resource and GISAID EpiFlu databases. Pairwise nucleotide identities were computed for assessing the evolutionary distance of H7N9 to other known avian and human viruses, and multiple sequence alignments with their position-specific entropy values were used in discussing how mutations on species-associated signature positions were introduced in the new H7N9 which may steer its way to human infection.
Results: This report analyzed the genomic characteristics of this new H7N9 virus. Nucleotide sequence analysis clearly reveals its origin from avian viruses. In this article, we particularly focus on its internal genes that are found to derive from H9N2—another subtype of avian influenza A virus which has been circulating in birds for years. Amino acid sequences at species-specific genomic positions were examined. Although the new virus contains mostly avian-like residues at these signature positions, it does contain several human-like signatures. For instance, at the position 627 of PB2, the new virus has human-characteristic K instead of

* Corresponding authors. Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, 259 Wenhua 1st Road, Kweishan, Taoyuan, Taiwan.
E-mail addresses: gwchen@mail.cgu.edu.tw (G.-W. Chen), srshih@mail.cgu.edu.tw (S.-R. Shih).

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avian-characteristic E; in addition, PB2-627K, PA-100A, PA-356R, and PA-409N are also human-like signatures in the new H7N9 virus.
Conclusion: The new H7N9 is an avian influenza A virus; however, it does harbor several human virus-like signatures, which raises great concern that it may have a higher probability to cross species barriers and infect humans.

Introduction

Influenza virus can infect various hosts, including avian and mammals. While it is generally true that there exists a species barrier to prevent avian influenza virus from infecting humans, sporadic human infections by avian influenza viruses have been continuously reported.[1] In particular, subtypes H5N1 and H7N7 of influenza A viruses have caused mortalities in humans.[2–4]

Influenza A viruses are enveloped negative-stranded RNA viruses with a segmented genome and are members of the Orthomyxoviridae family. There are eight RNA segments packaged in the viral core. Virus attacks cells through binding of the viral hemagglutinin (HA) to sialic acid-containing receptor. Upon leaving the infected cells, viral neuraminidase (NA) cleaves sialic acid to release its binding to HA. During its stay inside of the cells, virus utilizes its ribonucleoprotein (RNP) complex and cellular factors to replicate. Each RNP complex comprises an RNA strand wrapped by 3 polymerase proteins (polymerase basic 2, polymerase basic 1, and polymerase A, or PB2, PB1, and PA in short) and nucleoprotein NP. The RNP complex executes viral RNA transcription and replication in host nucleus, which provides opportunities to hijack resources from host cells.[5] Host proteins are generally assumed to be involved in the influenza A virus life cycle, and the lack of essential cellular proteins or the presence of inhibitory factors will influence the ability of virus to replicate. During the replication process, influenza A virus must overcome the host cellular microenvironment by affecting normal functions of cellular proteins.

A new subtype of avian influenza virus H7N9 emerged in China in March 2013, and growing number of fatal infections have been recorded since then.[6] This new virus emerged from reassortment of at least three subtypes of avian influenza viruses—its HA derived from H7N3, NA from H7N9 and all other six internal genes from H9N2.[6] In particular, the HA protein has a Q226L mutation, which is associated with increased binding to mammalian-like receptors bearing the alpha-2,6-receptor in the human upper airway.[7,8] Also found is the S31N mutation in the M2 protein, suggesting its resistance to the M2 channel blockers amantadine and rimantadine.[9,10] One key signature amino acid at 627 in its PB2 mutated to lysine, which is known to associate with mammalian adaptation and respiratory-droplet transmission of the highly pathogenic avian influenza virus H5N1.[7,11]

Large-scale scanning of influenza A viral genome sequences identified host-specific genomic signatures of human and avian influenza viruses.[12,13] These species-associated positions help in revealing the evolutionary preference of influenza viruses which is to attempt to destroy the barriers between the two host species. The introduction of genetic variations to viral RNP genes is considered one of the major determinants to overcome the species barriers.[5] In particular, three genetic substitutions K627E, N701D, and R591Q in the PB2 segment of influenza virus, have been reported to affect host cell tropism.[11,14,15] A single substitution of E (Glu) to K (Lys) at the PB2-627 position in avian influenza viruses significantly enhances polymerase activity,[16] viral replication,[17] transmission ability, and pathogenicity in mammalian cells and mice.[11,18]

This work analyzed and compared the genomes of H7N9 viruses isolated from fatal cases with those of other human and avian influenza A viruses. We particularly focus on the PB2 gene, due to the finding that the novel avian-origin H7N9 virus contains a human-like signature at position 627 of PB2, which has raised the concern that the new H7N9 virus can better adapt to humans.

Materials and methods

Nucleotide sequences and analysis

Nucleotide sequences for avian influenza A virus PB2 gene were downloaded from the Influenza Virus Resource (IVR) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html), including all-time 943 avian H5N1, 160 human H5N1, 287 avian H9N2, and five human H9N2 viruses. Human influenza A virus PB2 sequences were also obtained from IVR for comparative analysis. They include only recent Taiwanese strains—12 new H1N1 (Jan—Feb, 2008), 17 old H1N1 (Mar 2007—Dec 2008), and seven H3N2 viruses (Jan 2007—Jan 2008). All IVR sequences downloaded were full-length with redundancies removed. New H7N9 nucleotide sequences for three recent human cases were downloaded from the GISAID EpiFlu database (http://platform.gisaid.org). Pairwise sequence identity was computed by the global pairwise alignment program *needle* of EMBOSS package.[19] In particular, A/Shanghai/1/2013 was used as a reference sequence to compute the pairwise identities for the other PB2 sequences. MATLAB R2009b (The MathWorks, Inc., Natick, MA, USA) was used to produce the boxplots for these pairwise identities.

Protein sequences and analysis

Human and avian amino acid signatures for influenza A virus internal genes are based on Chen et al.[13] All full-length protein sequences for each of the influenza A virus internal genes were downloaded from IVR, without collapsing identical sequences. New H7N9 protein sequences for three recent human cases in China were downloaded from the GISAID EpiFlu database.

Results

Nucleotide sequence identities of the new H7N9 PB2 gene compared with other viruses

PB2 nucleotide sequence (2280-nt) of A/Shanghai1/2013 (1 of the 3 new H7N9 cases in China in April 2013) was used as a reference sequence to compute the sequence identities for other PB2 sequences. Fig. 1 shows the boxplots of these identities for each of the selected virus groups. It is observed that the three human virus groups all displayed relatively small median values (82.0—83.9% for recent Taiwanese new H1N1, seasonal H1N1, and seasonal H3N2). Their identity ranges were also narrow, apparently due to the limited virus populations we chose to sample. Nevertheless, Taiwanese new H1N1 viruses displayed slightly larger sequence identities to the new H7N9 PB2 among these three human viruses, reflecting the fact that PB2 of the new H1N1 in 2009 originated from the avian virus of North American lineage.[20]

While the range of identities also appeared narrow for the five human-isolated H9N2 viruses (all five in Hong Kong from 1997 to 1999), the median identity is 88.1%, which is clearly higher than in those Taiwanese human PB2 sequences. The median identity for human-isolated H5N1 viruses (86.5%) is also larger than those human viruses, and their range is bigger (85.4—88.4%) because of a much larger sample size (n = 160), time span (1997—2012) as well as geographic variation than those of human H9N2 cases. However, not only did avian H9N2 and avian H5N1 viruses exhibit larger median identities (87.1% and 86.3%, respectively), their ranges were also extensively stretched (81.4—99.2% for avian H9N2, and 83.4—95.7% for avian H5N1), apparently due to their large sampling sizes as well as intrinsic genetic diversities in various bird populations.

It is mentioned that the PB2 sequence varies only slightly among these three H7N9 cases (identities 99.6—99.9%). As a result, alternatively using either the other two (A/Shanghai/2/2013 or A/Anhui/1/2013) as a reference would not affect the sequence identity statistics reported above, nor produce any visible difference in Fig. 1.

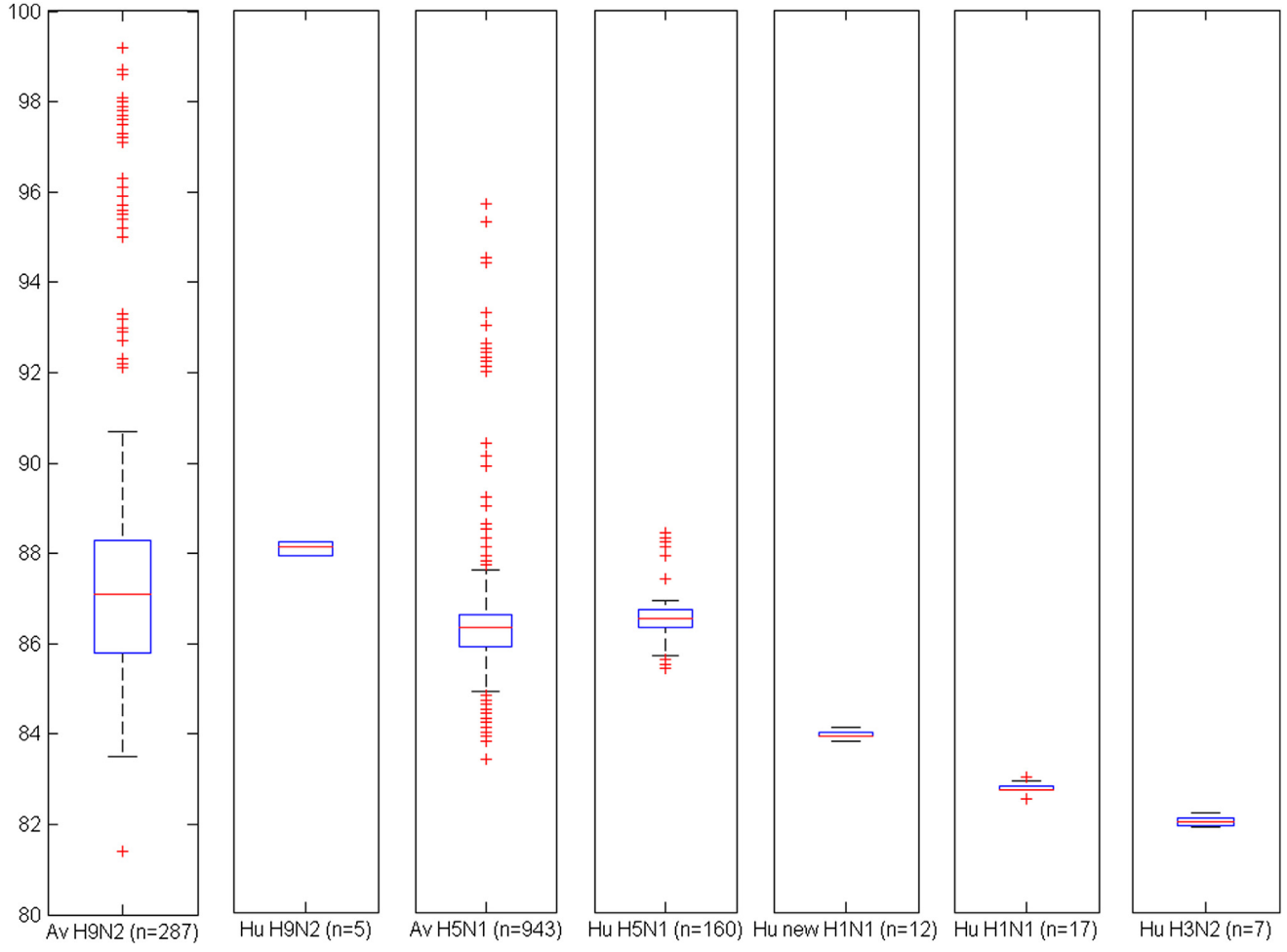

Figure 1 Boxplots for nucleotide sequence identities of influenza A PB2 in various virus groups, with respect to A/Shanghai/1/2013(H7N9). Median identities from left to right are 87.1, 88.1, 86.3, 86.5, 83.9, 82.7 and 82.0, respectively. Maximum identities from left to right are 99.2, 88.2, 95.7, 88.4, 84.1, 83.0 and 82.2, respectively. Minimum identities from left to right are 81.4, 87.9, 83.8, 85.4, 83.8, 82.5 and 81.9, respectively. Identities were computed by the program *needle* in EMBOSS package. Plots were produced by MATLAB 2009b. Av = avian, Hu = human; n = number of isolates.

Species-associated amino acid signatures of new H7N9

Chen et al utilized an entropy-based method and summarized 47 amino acid signatures for human and avian influenza A viruses, respectively.[19] Table 1 lists these positions, together with the amino acid compositions of the new H7N9 as well as all-time avian H5N1, human H5N1, avian H9N2, and human H9N2 viruses. Numbers included in the parentheses are the virus counts exhibiting one particular amino acid type. Forty-two of these signatures are avian-like for the new H7N9 viruses, reinforcing the earlier indications that the new virus is avian-originated.[6,21] It is noted that four signatures have already gone human-like, including PB2 627 from E to K, PA 100 from V to A, PA 356 from K to R, and PA 409 from S to N. Also characteristic to this new virus is a truncated NS1 of 217-aa, rather than the usually seen 230-aa in other viruses.

We also include both avian and human H9N2 signatures in Table 1 for assessing their potential of possessing human-like residues before the H9N2 viruses contributed their internal genes to the new H7N9 viruses.[6,21] It appears that all avian/human H9N2 viruses contain avian-like residues at these 47 signature positions, except that PB2 567 of five human cases all exhibited E, which is neither a human nor avian characteristic. Carefully examining the amino acid sequences for these avian H9N2 viruses, however, identified a number of signature positions which could potentially display a human-like residue. Consider PA-409, for example, while 250 of 342 avian H9N2 viruses investigated contain an avian-like residue S, there are 91 viruses (or 26.6%) displaying a human residue N. Nevertheless, this human-like residue also appears in the newly emerged H7N9 viruses. Another example is PA-356, which shows a human-like residue R in the new H7N9 viruses. Twelve of 342 (3.5%) H9N2 avian viruses are found to have R at this position in addition to the dominant avian-like K. More such human-residue-appearing positions are noticed in these avian H9N2 samples, although such appearances have not been observed as yet in the new H7N9 viruses. They are shown in boldface in Table 1, including PB2-44 and -702, PA-57, NP-214 and -372, M1-115 and -121, and M2-20.

Many humans were infected by avian H5N1 viruses since the first reported case in Hong Kong in 1997. This virus is highly pathogenic to both their avian hosts as well as to humans. Table 1 includes the amino acid statistics of avian and human H5N1 viruses. Although the primary residues are all avian-like for both of them, some human-like residues are seen as a secondary residue in avian H5N1 and found to stay in human H5N1 as we have observed in avian H9N2 viruses earlier. Examples include PB2-627, PA-404 and 409, and M1-137. However, some human-like residues appear only in avian and yet not (or less) in human cases. These include PB2-702, PB1-327, PA-100, NP-33 and -372, and M2-11 and -20.

PB2 627K appeared more in human cases

Special emphasis was placed on the species-associated PB2-627. Because H5N1 viruses have been circulating in wild birds and poultry for nearly two decades and human cases have continuously been reported, we analyzed 627 K/E for H5N1 which was isolated from avian sources versus humans. Table 2 summarizes the statistics of PB2 627 from 1047 avian H5N1, 177 human H5N1, 301 avian/human H9N2, and seven new H7N9 viruses. It is seen that human H5N1 has the highest K fraction of 27.68%, followed by avian H5N1 of 23.11%. This ratio is extremely small for all H9N2 PB2-627 (1.33%), suggesting their low pathogenicity to humans. Thus for all three published H7N9 genomes which showed K in PB2-627.

Discussion

HA, on the surface of the virus particle, sits in the cockpit which pilots the avian influenza A virus to other species, such as humans. It has been reported that position 226 is located at the receptor-binding site of HA protein, and a change from Q to L at this position in an H5N1 virus would increase its binding affinity to 2-6 linked sialic acid, which is a sugar in the receptors of human respiratory epithelium.[22] Although the first H7N9 isolate in China—A/Shanghai/1/2013, still contained a 226Q, a number of subsequent isolates (A/Shanghai/2/2013, A/Anhui/1/2013, A/Zhejiang/DTID-ZJU01/2013) were found to have a Q226L substitution. This raised the concern that the new H7N9 would soon be capable of transmission to humans. A 5-aa deletion in the stalk region of NA protein was also noticed in this new virus, which is similar to the 19-aa deletion of the NA stalk in H5N1. Although it is still unclear how such genetic alteration may be involved in human transmission, it is reported that the length of stalk may be associated with viral virulence.[23]

This study focuses on the internal genes of influenza A viruses, which are important for viral replication and virulence. It has been reported that the new H7N9 virus acquired its internal genes from H9N2 virus. Our analysis shows that, among those 47 signature positions that separated human and avian viruses in these internal genes, PB2-627, and PA-100, -356 and -409 have already become human-like. In particular the drift of PB2-627 from E to K has been considered a major mutation for an avian virus to adapt to mammalian species. From Table 1, however, we see very few cases of PB2-627K (4 of 296 avian H9N2 viruses) and PA-100A (2 of 342 avian H9N2 viruses) in hundreds of our collected H9N2 samples. However, human-like residues are seen in PA-356 and 409 more frequently. For example, human-like residue N is seen in PA-409 in 26.6% of the avian H9N2 population. Since only limited number of H7N9 isolates are available at present, it is speculated that the positions carrying more human-like residues in H9N2 viruses may possess the potential to emerge in the new H7N9 viruses, for example, PB2-44 to S, PB2-702 to R, NP-214 to K, NP-372 to D, etc. Certainly we cannot rule out the possibility for other human-like residues to appear in the new H7N9 viruses. Two such examples would be PB2-627 from E to K and PA-100 from V to A in H7N9, in both cases only very limited human-like residues were observed in their ancestral H9N2 isolates.

Fig. 1 shows that avian H9N2 and avian H5N1 displayed an overall higher PB2 sequence identity than the others. In particular, 29 avian H9N2 viruses are more than 95% identical

Table 1 Amino acid compositions at 47 species-associated signature positions for H5N1, H9N2, and H7N9 viruses.

Gene	Pos	Av	Hu	Avian H5N1	Human H5N1	Avian H9N2	Human H9N2	New H7N9
PB2	44	A	S	A(1046), S(1)	A(177)	A(279), **S(16)**, T(2)	A(5)	A(3)
	199	A	S	A(1044), T(2), S(1)	A(170), S(7)	A(297)	A(5)	A(3)
	271	T	A	T(1043), M(3), A(1)	T(174), M(2), A(1)	T(292), M(4), I(1)	T(5)	T(3)
	475	L	M	L(1041), M(6)	L(176), F(1)	L(296), W(1)	L(5)	L(3)
	567	D	N	D(1041), E(5), N(1)	D(160), E(17)	D(279), E(18)	E(5)	D(3)
	588	A	I	A(924), T(112), V(10), I(1)	A(172), T(5)	A(239), T(26), V(25), I(7)	A(5)	A(3)
	613	V	T	V(1039), A(8)	V(175), A(2)	V(293), A(2), I(2)	V(5)	V(3)
	627	E	K	E(805), **K(242)**	E(128), **K(49)**	E(240), V(51), K(4), G(1)	E(5)	**K(3)**
	702	K	R	K(1031), **R(15)**, T(1)	K(172), R(5)	K(249), **R(48)**	K(5)	K(3)
PB1	327	R	K	R(1153), **K(23)**, W(1)	R(163), K(2)	R(322)	R(5)	R(3)
	336	V	I	V(1170), I(5), A(1)	V(165)	V(315), I(5), A(2)	V(5)	V(3)
PA	28	P	L	P(1231), L(2), T(2), S(1)	P(179)	P(342)	P(5)	P(3)
	55	D	N	D(1235), N(1)	D(179)	D(340), N(2)	D(5)	D(3)
	57	R	Q	R(1228), Q(7), W(1)	R(179)	R(325), **Q(15)**, K(2)	R(5)	R(3)
	100	V	A	V(1197), **A(20)**, I(18), F(1)	V(143), I(36)	V(311), I(27), A(2), D(2)	V(5)	**A(3)**
	225	S	C	S(1233), G(1), N(1), R(1)	S(179)	S(341), C(1)	S(5)	S(3)
	268	L	I	L(1232), F(2), I(1), V(1)	L(179)	L(339), I(2), P(1)	L(5)	L(3)
	356	K	R	K(1231), R(3), I(1)	K(178), R(1)	K(330), **R(12)**	K(5)	**R(3)**
	404	A	S	A(939), **S(288)**, T(5), P(3)	A(134), **S(44)**	A(339), T(2), S(1)	A(5)	A(3)
	409	S	N	S(1215), G(10), **N(10)**, R(1)	S(163), **N(16)**	S(250), **N(91)**, R(1)	S(5)	**N(3)**
	552	T	S	T(1166), A(70)	T(179)	T(390), N(2)	T(5)	T(3)
NP	16	G	D	G(1131), S(36)	G(166), S(3)	G(345), D(2), S(2)	G(5)	G(3)
	33	V	I	V(793), **I(373)**, A(1)	V(169)	V(348), I(1)	V(5)	V(3)
	61	I	L	I(1164), M(3)	I(168), L(1)	I(346), L(2), M(1)	I(5)	I(3)
	100	R	V	R(1162), K(5)	R(168), I(1)	R(344), K(5)	R(5)	R(3)
	109	I	V	I(1161), V(4), T(2)	I(169)	I(344), S(2), T(2), V(1)	I(5)	I(3)
	214	R	K	R(1164), K(3)	R(169)	R(322), **K(26)**, L(1)	R(5)	R(3)
	283	L	P	L(1166), P(1)	L(168), P(1)	L(347), P(2)	L(5)	L(3)
	293	R	K	R(1161), K(6)	R(169)	R(349)	R(5)	R(3)
	305	R	K	R(1167)	R(169)	R(346), S(2), K(1)	R(5)	R(3)
	313	F	Y	F(1154), S(12), L(1)	F(169)	F(349)	F(5)	F(3)
	357	Q	K	Q(1159), K(6), R(2)	Q(168), K(1)	Q(347), K(2)	Q(5)	Q(3)
	372	E	D	E(1143), **D(24)**	E(169)	E(326), **D(23)**	E(5)	E(3)
	422	R	K	R(1167)	R(169)	R(348), K(1)	R(5)	R(3)
	442	T	A	T(1167)	T(169)	T(349)	T(5)	T(3)
	455	D	E	D(1166), E(1)	D(169)	D(346), E(3)	D(5)	D(3)
M1	115	V	I	V(1846), G(1), I(1)	V(199)	V(835), **I(11)**	V(5)	V(3)
	121	T	A	T(1847), A(1)	T(198), S(1)	T(804), **A(41)**, N(1)	T(5)	T(3)
	137	T	A	T(1830), **A(18)**	T(149), **A(50)**	T(841), A(5)	T(5)	T(3)
M2	11	T	I	T(1250), **I(13)**, S(9)	T(170)	T(359), I(3), S(1)	T(5)	T(3)
	20	S	N	S(1225), I(18), **N(18)**, R(11)	S(123), I(46), N(1)	S(330), K(16), **N(16)**, G(1)	S(3), R(2)	S(3)
	57	Y	H	Y(1271), N(1)	Y(170)	Y(360), H(3)	Y(5)	Y(3)
	86	V	A	V(1266), I(5), A(1)	V(169), A(1)	V(361), A(1), S(1)	V(5)	V(3)
	93	N	S	N(1249), D(17), H(3), Y(2), S(1)	N(170)	N(362), S(1)	N(5)	N(3)
NS1	81	I	M	I(139), M(3), Gap(1778)	I(19), Gap(213)	I(745), V(27), A(5), T(5), M(3)	I(6)	I(3)
	227	E	R	E(1840), G(55), K(12), R(1), S(1), Gap(26)	E(212), G(12), S(1), Gap(7)	E(214), K(84), G(20), R(1), Gap(466)	E(5)	Delete
NS2	107	L	F	L(1403), Gap(1)	L(195)	L(608), P(2)	L(6)	L(3)

Residues in bold-and-underlined are human-like signatures; residues highlighted are neither human- nor avian-like signatures.

to A/Shanghai/1/2013, with the top five as A/brambling/Beijing/16/2012 (99.2%), A/chicken/Zhejiang/329/2011 (98.7%), A/chicken/Shanghai/C1/2012 (98.6%), A/duck/Shanghai/C164/2009 (98.1%) and A/chicken/Jiangsu/Q3/2010 (98.0%). Co-incidentally, bramblings are migratory birds that are widespread throughout the forests of northern Asia, and the other four isolates were from commonly seen poultry in either Shanghai or nearby areas (Zhejiang and Jiangsu) as

Table 2 PB2 627 amino acid composition for H5N1, H9N2, and H7N9 viruses.

Residue	Avian H5N1	Human H5N1	Avian/Human H9N2	Human H7N9
E	805	128	245	0
K	**242**	**49**	**4**	**3**
V	0	0	51	0
G	0	0	1	0
Total	1047	177	301	3
K Percentage	23.11%	27.68%	1.33%	100.00%

early as 2009. This demonstrates a high correlation between the new H7N9 PB2 gene and the recently circulated avian H9N2 viruses, based on both ecology (migratory birds) as well as geographic locations (greater Shanghai area). This observation reinforces the importance of continuously monitoring the avian influenza virus genomes in these areas, both in domestic poultry as well in migratory birds.

Although all the available cases of new H7N9 feature PB2-627K, their potential for disrupting the species barrier for human-to-human infection remains to be investigated. Although its PB2 was derived from H9N2, we showed that only four cases of PB2-627K were detected in all H9N2 PB2 sequences we have investigated. It is not clear how this seemingly sporadic PB2-627K cases in H9N2 population had triggered the recently reported fatal cases. In Tables 1 and 2 we observed the association of human-like residues between avian and human H5N1 viruses. Such inference is statistically possible, thanks to the effort of global surveillance of H5N1 viruses in the past decade. We have already seen that the new H7N9 isolated from human is of avian origin, and already contains several human like signatures in its internal genes. It is likely that H7N9 will evolve by taking a similar path to that of the H5N1 virus in the past. Although the genetic outfits summarized in Table 1 for H9N2 viruses may provide certain clues of how the internal genes for the new H7N9 may change, continuing to monitor new H7N9 as well as related avian viruses (such as H9N2) would be critical to better assess the potential for the virus to cross the avian-human species barrier. If the current H7N9 epidemic is not controlled soon, a future possible pandemic may be eminent and then a good rapid test assay[24] and effective vaccine would be needed.

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