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Glycan Receptor Binding of the Influenza A Virus H7N9 Hemagglutinin

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SUMMARY

The advent of H7N9 in early 2013 is of concern for a number of reasons, including its capability to infect humans, the lack of clarity in the etiology of infection, and because the human population does not have pre-existing immunity to the H7 subtype. Earlier sequence analyses of H7N9 hemagglutinin (HA) point to amino acid changes that predicted human receptor binding and impinge on the antigenic characteristics of the HA. Here, we report that the H7N9 HA shows limited binding to human receptors; however, should a single amino acid mutation occur, this would result in structural changes within the receptor binding site that allow for extensive binding to human receptors present in the upper respiratory tract. Furthermore, a subset of the H7N9 HA sequences demarcating coevolving amino acids appears to be in the antigenic regions of H7, which, in turn, could impact effectiveness of the current WHO-recommended prepandemic H7 vaccines.

INTRODUCTION

In late March of 2013, an H7N9 influenza A virus subtype was identified that was found to infect humans, causing rapidly progressing severe lower respiratory tract infection and mortality (Gao et al., 2013). As of early May, 131 reported cases have been confirmed, with most cases in mainland China. Detailed sequencing and analysis of the human isolates of H7N9 have offered insights into their potential origin and factors that govern the virus’s virulence and pathogenicity (Li et al., 2013). The transmission and pathogenicity associated with this virus is unanticipated for several reasons. First, transmission of H7 viruses from birds to mammals has been reported only rarely (Kwon et al., 2011). Additionally, until the advent of H7N9, human infections with N9 subtype viruses have not been previously reported. Finally, human infections with other H7 viruses (primarily H7N2, H7N3, and H7N7), even with high-pathogenicity viruses containing a polybasic cleavage site in HA, have primarily resulted in conjunctivitis or uncomplicated illness, with few exceptions (Fouchier et al., 2004; Hirst et al., 2004).

Analysis of newly arising H7N9 strains, including A/Shanghai/1/2013, A/Shanghai/2/2013, and A/Anhui/1/2013, indicates that H7N9 is a reassorted virus incorporating envelope genes from at least two H7 strains (hemagglutinin [HA] from an H7N3 strain and neuraminidase [NA] from an avian-adapted H7N9 strain) with the internal genes from at least two H9N2 avian-adapted influenza strains (Li et al., 2013). Further analysis of the H7N9 gene segments has shown the occurrence of signature amino acids associated with adaptation to human host and virulence (summarized in Table 2 in Gao et al. [2013]). H7N9 strains exhibit hallmark mutations that are thought to correlate with increased virulence and potentially transmission in animal models such as mice and ferrets, including the E627K mutation in PB2 (which is known to play a key role in human-to-human respiratory droplet transmission [Van Hoeven et al., 2009]). Furthermore, genetic analysis indicates the presence of mutations, in at least some strains, within the M2 ion channel and NA that confer drug resistance to the adamantanes and oseltamivir, respectively.

In contrast to the above analysis, several of the hallmark features found in highly pathogenic influenza strains (e.g., H5N1), including the N66S mutation in PB1-F2, the aforementioned polybasic sequence in the linker between HA1 and HA2, and the PDZ-binding motif in C terminus of NS1, are absent in the H7N9 human isolates analyzed to date. Within this context and given the somewhat confounding genetic and epidemiological evidence of the relative human-adaptation of H7N9, one of the most important outstanding questions is the “status” of the HA protein for these isolates. Characterization of the HA protein is important given its role in virulence (Pappas et al., 2008) and virus neutralization to pre-existing antibodies through antigenic memory (Hensley et al., 2009). Finally, the receptor-binding properties of HA, governing a given virus’s tissue and organismic tropism, are one of the key factors that critically govern aerosol transmissibility, including human-to-human transmission (Van Hoeven et al., 2009).

Previous studies have demonstrated that one of the key properties governing human adaptation of influenza A virus is a “switch” in the glycan receptor-binding specificities of viral HA (Skehel and Wiley, 2000). Therefore, together with hallmark mutations in other genes, such as PB2, describing mutations in

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HA that lead to such a "switch" becomes important for surveil-

lance purposes. From the standpoint of human tissue tropism,
the HA from human-adapted viruses, including pandemic
strains, shows extensive binding to the apical surface of human
upper respiratory tissues (such as trachea) and also shows char-
acteristic binding to mucin-secreting nonciliated goblet cells on
the apical surface and to submucosal glands in ferret respiratory
tract (Matrosovich et al., 2004; Nicholls et al., 2007; Srivin-
asan et al., 2008). Through lectin staining, it has been demonstrated
previously that these regions in human tracheal sections pre-
dominantly display diverse glycan receptors terminated by
α2→6 sialic acid linkage (human receptors) (Srinivasan et al.,
2008; Viswanathan et al., 2010). We have previously demon-
strated with H1, H2, and H3 subtypes that this binding property
of human-adapted viruses is one of the key factors that corre-
lates with their ability to efficiently transmit via respiratory drop-
lets in ferrets—a well-established animal model to measure the
potential for airborne human-to-human transmission (Jayara-
man et al., 2011; Maines et al., 2009).

In the case of H7N9, based on the presence of a leucine resi-
due in the 226 position (H3 numbering) of the HA, earlier studies
have predicted that this HA would have strong binding to human
receptors (Gao et al., 2013; Liu et al., 2013). These recent studies
note that HA-glycan receptor interaction is a critical property of
the virus and that experimental characterization of this property
for the H7N9 subtype is important.

Here, we report the molecular and structural features of the
glycan receptor-binding site (RBS) of H7N9 HA and the experi-
mental characterization of its binding to physiological glycan
receptors in the human respiratory tract. Contrary to the pre-
dicted strong binding to human receptors, H7N9 HA shows
limited binding to these receptors in the human upper respira-
tory tract when compared to human receptor binding of other
human-adapted HAs. The experimentally observed limited bind-
ing to human receptors by H7N9 HA is consistent with the
analysis of its RBS structural features. The structural and se-
quence analyses further point to a single Gly226→Ser amino
acid change, which modifies the network of interresidue con-


RESULTS

Given that H7N9 is a newly emerged subtype, there is a limited
set of HA sequences available for this subtype to do a compre-
hensive analysis of sequence evolution of its RBS. Therefore, in
order to understand glycan receptor-binding properties of H7N9
HA, we chose a representative human isolate A/Anhui/1/2013
(Anh13). Because there is no crystal structure available, we con-
structed a homology-based structural model of Anh13 HA and
compared its RBS with H3 HA (its phylogenetically closest
human-adapted HA [Figure S1 available online]). Anh13 was
chosen because it shares substantial sequence identity with
many other reported strains of H7N9, including A/Shanghai/2/
2013 and A/Hangzhou/1/2013. Additionally, as noted previously
(Gao et al., 2013), although there are changes in the HA
sequence of Anh13 compared to the HA sequence of other
reported H7N9 strains, including A/Shanghai/2/2013, the HA of
Anh13 contains a key Q226L mutation that has been reported to
be important for altered receptor specificity for group 2
viruses, including H3 and H7. As such, Anh13 (and related
viruses) are more likely than viruses such as A/Shanghai/2/
2013 to be of concern from the standpoint of altered receptor
specificity and, hence, human transmissibility.

Earlier, we defined a framework that incorporated descriptors
of the structural topology of the human glycan receptor as well as
interamino acid interaction networks within the RBS to define
the molecular features of the RBS of H5 HA for high-avidity/
specificity binding to human glycan receptors (Chandrasekaran
et al., 2008; Soundararajan et al., 2011). We therefore compared
the molecular features of the Anh13 RBS with those of H3 HA
(from A/Aichi/1/68 or Aich68, a strain from the 1967–1968
pandemic), which was recently cocrySTALLized with both avian
and human receptors (Lin et al., 2012). Also of importance,
because Aich68 represents a human-adapted virus, comparison
of its HA to that of Anh13 provides an important benchmark to
address the question of whether the HA from Anh13 shares
structural characteristics with HAs of human-adapted viruses
and, if not, which structural characteristics are missing.

Structural analyses of both H7 and H3 HAs indicate that
the structural topology of the human glycan receptor bound in
the RBS of HA is such that it has a clearly defined base region
consisting of the terminal Neu5Acα2→6Galβ1→ motif and an
extension region consisting of at least a disaccharide
→4GlcNAcβ1→3Galβ1→. On the other hand, the topology of
the avian receptor bound within the HA RBS is such that majority
of the contacts with residues within the RBS involve the terminal
Neu5Acα2→3Galβ1→ motif in the base region (Figure 1).
The 130 (residues 131–138), 140 (residues 140–145) and 220 (resi-
dues 219–228) loops in the RBS make critical contacts with the
disaccharide motif in the base region and thus play a key role
in dictating the avian or human receptor-binding preference
(residue position numbering is based on Aich68 HA-glycan
cocrystal structure [Protein Data Bank ID: 2YPG]). Additionally,
residues within the 190 helix (residues 190–196) and a part of
the 150 loop (residues 156–160), if properly positioned, make
critical contacts with the extension region of the human receptor.
Taken together, these five loops and one helix in the RBS that
make contact with the base and potentially with the extension
region of the human receptor and their network of interactions
with spatially proximal residues in the RBS constitute a complete
set of molecular features that should be analyzed to understand
the receptor-binding preference of an HA. Comparison of these
Figure 1. Structural HA-Glycan Receptor Complexes

(A) Structural model of Anh13 H7 HA RBS in complex with avian receptor. The avian receptor is shown as a stick at 40% transparency with carbon atom colored in blue. The loop and helix regions used to define molecular features of the HA RBS are shown. The side chains of the key amino acids in these regions that make contact with the glycan receptor are shown. The side chain of Q at the 226 position observed in all other H7 HAs (prior to H7N9 outbreak) is shown as stick with 40% transparency (carbon atom colored green). The networks of interresidue interaction contacts are shown as two-dimensional maps near the corresponding residue positions. The map is composed of circular nodes representing the amino acid positions (which are labeled above the nodes) and is colored according to the degree of interresidue contact (lightest shade of red indicating lowest contact and darkest shade of red indicating highest contact).

(B) The structural model of Anh13 H7 HA RBS in complex with human receptor (shown as a stick with 40% transparency and carbon atom colored in orange).

(legend continued on next page)
The key amino acids in the 130, 140, 150, and 220 loops and 190 helix that bind to the base and extension region of the glycan receptors in Aichi68 and Anh13 are shown. The 150 loop has amino acids (positions 153 and 155) that are involved in contacts with the sialic acid in the base region and those that are involved in binding to extension region. See also Figure S3.

To validate the structural analyses, we analyzed binding of Anh13 to tissue sections, representative of the human respiratory tract, which display the physiologically relevant receptors for influenza A viruses (Figure S2A). Anh13 stained the apical surface and submucosal region of the human trachea, which express glycans known to be receptors of human-adapted viruses (Jayaraman et al., 2012; Shinya et al., 2006). However, the intensity and the extent of tracheal apical surface staining by Anh13 were substantially lower than what is typically observed for human-adapted HAs (Figure S2A). Furthermore, Anh13 HA also showed minimal binding to deep-lung alveolar section—a region that is extensively stained by avian-adapted HAs (Figure S2B). In contrast, the G228→S mutant HA showed a dramatic and significant increase in the extent and intensity of staining to apical surface of the tracheal section, including extensive staining of nonciliated goblet cells in a fashion similar to that of other human-adapted HAs and *Sambucus nigra* agglutinin I (SNA I) (Figures 2 and S2C). Interestingly, the G228→S mutation also substantially increased its binding to the alveolar sections (Figure S2B). These results are consistent with the structural analyses of the RBS features of H7N9 HA.

Finally, in the context of H7N9 evolution, two mutations (174S and 226L) appear to be unique to the H7N9 HA sequences. As noted above, the residue at 226 is a critical determinant of the receptor-binding specificity of H7 HA, with human viruses favoring L/I and avian viruses favoring Q. These two positions are also part of a larger cluster of coevolving positions (122A, 174S, 186V, 202V, and 226L), all within the 50–230 HA region, which demarcates the virus from its previous H7 ancestors (see the Experimental Procedures). The above observations indicate that the H7N9 HA has evolved to be distinct from its predecessors. Previous H7 strains carrying single mutations (from the coevolving features between the Anh13 and Aichi68 HA shows many similarities, as well as some important differences (Table 1). First, many of the residues in the 130 loop, 140 loop, and 220 loop are similar between the HA of Anh13 and Aichi68. Based on this level of similarity, we find that the network of interresidue contacts involving these residues is also similar (Figures 1A and 1C). The key structural differences noted in this analysis include part of the 190 helix and the 150 loop required for contact with the extension region of the human receptor. In the Aichi68 HA, the residues interacting with the extension region include Q189, S193, K156, and S159 (Figure 1C), whereas, in H7 HA, these residues include K193, T158, D159, and N160 (Figure 1B). Furthermore, in the case of the H7 HA, R131 is positioned to make an additional contact with the extension region (Figure 1B). In addition to the differences in the amino acids at these positions—and also of note—are differences between Aichi68 and Anh13 in the interresidue interaction network governed by the residue at position 228. This position is a Ser in Aichi68 but is a Gly in Anh13. The S228 position in H3 is critical for the inter-amino acid network involving S186, T187, and E190, which positions E190 to make critical contacts with the sialic acid of both avian and human receptors (Figure 1C). On the other hand, G228 in H7 HA does not possess this interamino acid network and, therefore, the network of interresidue contacts involving E190 in H7 is different from that of H3 HA and instead includes 193 (and its network) and 189 (Figure 1A).

Extending this analysis, the E190 in H7 HA is positioned to make additional contacts with the extension region instead of the critical contact with the sialic acid in the base region (Figure 1B). Therefore, Anh13 H7 HA lacks at least two critical contacts involving the 190 and 226 HA positions with the Neu5Ac2→3Galβ1→ terminal motif as observed in avian-adapted HAs. In the case of human receptor contacts, Anh13 has lower contacts with the base region owing to the absence of the S228 residue. Our structural analysis pointed to the H7N9 HA having a substantially lower binding to avian receptors than typical avian-adapted Has, as well as lower binding to human receptors than the human-adapted H3 HAs. Furthermore, the RBS of H7N9 HA is such that a single G228→S amino acid change would modify the interamino acid network in the RBS to position the E190 and S228 residues for optimal contacts with both avian and human receptors.

Table 1. Amino Acid Differences in the RBS of Aichi68 H3 and Anh13 H7 HAs

<table>
<thead>
<tr>
<th></th>
<th>Base (Neu5Acα2→3Galβ1→)</th>
<th>Extension (→4GlcNAcβ1→3Galβ1→4→)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>130 loop</td>
<td>140 loop</td>
</tr>
<tr>
<td></td>
<td>150 loop</td>
<td>150 loop</td>
</tr>
<tr>
<td></td>
<td>190 helix</td>
<td></td>
</tr>
<tr>
<td>Aichi68</td>
<td>T  N  S  A  S  S  P  W  G  L  S  S  W  T  K  S  G  S  T  S  T  N  Q  E  T  S  L  V</td>
<td></td>
</tr>
<tr>
<td>Anh13</td>
<td>R  N  T  A  S  A  P  Q  G  L  S  G  W  L  S  T  D  N  V  S  T  A  E  T  K  L  G</td>
<td></td>
</tr>
</tbody>
</table>

(C) Aichi68 H3 HA RBS-human receptor complex as observed in the X-ray cocrystal structure (PDB ID: 2YPG). The interresidue interaction network of the key RBS residues is similar to what was shown in (A). See also Figure S1. Note the similarities in the network of residues in the 130, 140, and 220 loop between the H3 and H7 HA (expanded in Table 1). On the other hand, the network involving residues in the 190 helix is quite different, and this difference is brought about by the amino acid differences and also by the Gly in H7 HA versus Ser in H3 HA in the 228 position.

(D) The structural model of the G228→S mutant of Anh13 H7 HA RBS in complex with human receptor. Note that the network involving residues in the 190 helix in the mutant is more similar to that observed in H3 HA than the WT. The interresidue contact networks that are different between the mutant and WT HA are shown in red dotted circle. See also Figures S1 and S3.
cluster) are predominantly from the Eurasian lineage, suggesting that viruses from this lineage have higher potential to generate variants when compared to the American lineage (Figure S3). Nucleotide analyses of the RBS-proximal region of HA (residues 50–230 of HA1) of Eurasian sequences from 1902 to 2013 show strong diversifying (positive) selection at 156 (see the Experimental Procedures). The same position has been shown to be under selection pressure in the H1 subtype as well (Li et al., 2011).

Previously, we defined a quantitative metric to compare the antigenicity of two HAs. Briefly, the metric, called antigenic intactness (AI), is directly proportional to the fraction of residues conserved in the immunodominant antigenic sites between two HAs. In our previous work, we showed good agreement between AI values and antigenic relatedness metric computed from ferret antiserum HA inhibition (HI) cross-reactivity data (Tharakaraman et al., 2013), indicating that AI values could be applied to predict vaccine-induced cross-reactive antibody responses. Critically, strains that are antigenically related had AI > 80%, whereas strains that are not related to each other had AI < 80% (Tharakaraman et al., 2013). Keeping 80% as the cutoff, the AI values between the recent World Health Organization (WHO)-recommended H7 vaccines strains (A/Canada/rv444/2004 [H7N3], A/mallard/Netherlands/12/2000 [H7N3], and A/New York/107/2003 [H7N2]) and the A/Anhui/1/2013 H7N9 HA were computed. The H7N9 HA has AI values of 67%, 89%, and 70% with A/Canada/rv444/2004 (H7N3), A/mallard/Netherlands/12/2000 (H7N3), and A/New York/107/2003 (H7N2), respectively, suggesting that only A/mallard/Netherlands/12/2000 (H7N3) may be effective as a vaccine component. However, amino acid differences between A/Anhui/1/2013 (H7N9) and A/mallard/Netherlands/12/2000 (H7N3) at key antigenic sites (122 in site A and 188 and 189 in site B) might limit this mallard strain to be effective.

In the context of the Q228S mutation and given that H7N9 is a subtype that has just emerged in the human population, it is difficult to predict whether this mutation alters antibody response to this region in this subtype. However, it is generally known that the 220 loop, which includes the 228 position, is an antigenic site for some HA subtypes (224 and 225 are part of antigenic site Ca in H1 subtype; 222 is part of antigenic site I-C in H2 subtype; and 220 is part of antigenic site D in H3, a subtype that is phylogenetically closest to H7); thus, antibody response targeting this region in H7N9 cannot be excluded. As such, it will be important to complete serological analysis to confirm the vaccine implications of our AI analyses.

DISCUSSION

The emergence of an H7N9 influenza A virus subtype poses a significant global health concern, given that it has led to severe infection and mortality in humans. Although preliminary genetic analysis of this subtype has led to predictions about its human host adaptation based on hallmark genetic signatures, including strong binding to human receptors, this virus has not yet caused widespread infection in humans resulting from aerosol human-to-human transmission. Earlier studies have highlighted the importance of determining the glycan receptor-binding property of H7N9 HA because this property is one of the many key factors.
that govern human adaptation of the virus (Gao et al., 2013; Liu et al., 2013). Therefore, in this study, we experimentally characterized glycan receptor-binding properties of H7N9 HA to assess the human adaptation of this HA. To the best of our knowledge, this is the first report on the glycan binding data of the H7N9 HA.

Our structural analyses and binding of H7N9 HA to human respiratory tissues demonstrate that it possesses a distinct binding tropism when compared to either an avian-adapted or a human-adapted HA. Our findings shed light on the distinct tropism of this H7N9 HA that is contrary to the expected strong human receptor-binding preference predicted from earlier sequence analyses (Gao et al., 2013; Liu et al., 2013). This distinct tropism would likely impinge on the aerosol transmissibility of the H7N9 viruses in ferrets when compared to the efficient transmission observed in the past pandemic viruses. The limited human and avian receptor-binding of the H7N9 HA raises a question as to whether this currently circulating subtype is an intermediate in the adaptation to the human host. Answering this question at this point in time is limited by the availability of sequence information for this recently emerged subtype. Nevertheless, our structural and experimental analyses point to an important role for G228S amino acid change in the RBS, should it emerge in the current H7N9 HA, in substantially increasing its binding to human receptors in the human respiratory tract. Of note—within the context of this study—is the increased binding of the mutant HA to the nonciliated goblet cells in the human trachea. This is significant because binding to goblet cells is one of the hallmarks of human-adapted HA s (Jayaraman et al., 2012; Matrosovich et al., 2004; Srinivasan et al., 2008). Even in cultures of differentiated human airway epithelial cells, the human influenza A viruses are found to predominantly infect nonciliated cells as compared to avian influenza A viruses, which target the ciliated cells (Matrosovich et al., 2004).

Previously, several groups, including us, have studied the receptor specificity of influenza HA using “prototypic” glycan arrays containing limited sets of glycans capped with α2–3 or α2–6 linked sialic acid. We find that, in most cases with H1, H2, and H3 HA, the observed binding on glycan array correlated to observed binding of these HAs on physiological glycans from respiratory tract tissue sections. However, as noted in our previous study of HA from H7N2 A/Netherlands/219/2003, we observed that introduction of 226L and 228S resulted in significant binding to human receptors in the human respiratory tract. The significance of the change in residue 156 in receptor binding or other HA function is unclear, although the neighboring G228S change in dramatically facilitates monitoring the evolution of H7N9, including the acquisition of amino acid changes such as G228S that would make it closer to human adaptation. Our study warrants further investigation of introducing this single amino acid change to H7N9 virus in the context of other genetic changes characteristic of human-adapted viruses (such as K627 in PB2) and studying its transmission properties in ferrets. This analysis therefore sets the stage for future in vitro studies in human respiratory tract cell cultures and in vivo studies in ferret and mice to investigate the replication potential, virulence, pathogenicity, and respiratory droplet transmission using these recombinant WT and mutant HA H7N9 viruses. Taken together, these findings have important implications for surveillance of H7N9 mutations in clinical settings, as well as for vaccine development efforts.

**EXPERIMENTAL PROCEDURES**

**Cloning, Baculovirus Synthesis, and Mammalian Expression and Purification of HA**

Anh13 WT and G228→S mutant HA sequences were codon optimized for mammalian expression, synthesized (DNA2.0, Menlo Park, CA), and subcloned into modified pcDNA3.3 vector for expression under CMV promoter. Recombinant expression of HA was carried out in HEK293-F FreeStyle suspension cells (Invitrogen, Carlsbad, CA) cultured in 293-F FreeStyle Expression Medium (Invitrogen, Carlsbad, CA) maintained at 37°C, 80% humidity, and 8% CO2. Cells were transfected with Polyethylene-imine Max (PEI-MAX, PolySciences, Warrington, PA) with the HA plasmid and were harvested 7 days postinfection. The supernatant was collected by centrifugation, filtered through a 0.45 μm filter system (Nalgene, Rochester, NY), and supplemented with 1:1,000 diluted protease inhibitor cocktail (Calbiochem filtration and supplemented with 1:1,000 diluted protease inhibitor cocktail (EMD Millipore, Billerica, MA)). HA was purified from the supernatant using His-trap columns (GE Healthcare) on an AKTA Purifier FPLC system. Eluting fractions containing HA were pooled, concentrated, and buffer exchanged into 1x PBS (pH 7.4) using 100 kDa MWCO spin columns (Millipore). The purified protein was quantified using the BCA method (Pierce, Rockford, IL).
Homology Modeling of HA

A structural model of Anh13 HA was built using the MODELER homology modeling software. The crystal structure of A/ Netherlands/219/2003 (Neth03) HA (PDB: 4DJ8) was used as a template to build the model. The structural model of Anh13 bound to avian receptor was constructed by superimposing the HA1 from cocrystal structure of Neth03-avian receptor complex (PDB ID: 4DJ7) on Anh13 HA1. The structural model of Anh13 in complex with human receptor was constructed by superimposing the HA1 from cocrystal structure of Aich68-human receptor complex (PDB ID: 2YPG) with HA1 of Anh13. The final models were subjected to energy minimization (500 steps conjugate gradient + 500 steps steepest descent) with potentials assigned using AMBER force field.

Coevolution, Phylogeny, and Selection Analyses of H7 HA Sequences

A total of 625 nonredundant full-length H7 HA sequences were downloaded from GISAID. To further eliminate redundancy, the sequences were grouped according to subtype, host, year, and country, and a representative sequence was chosen from each group. This led to a total of 231 HA sequences. Coevolving groups of amino acids were predicted using the CAPS online server for protein coevolution (http://bioint.gr/tcd.ie/caps/). The results indicate functionally or structurally linked regions that are subjected to strong selective constraints. A phylogeny tree was constructed from the 231 HA sequences using the neighbor-joining method found in MEGA 5.1 software (http://www.megasoftware.net/). Protein-coding nucleotide sequences were extracted for the 114 Eurasian HA sequences, and the region-encoding invariant physiological target site for human-adapted influenza A viruses (Imai et al., 2012; Jayaraman et al., 2012; Mansfield, 2007; Shinya et al., 2006). The apical surface and submucosal regions of the human trachea have been shown to predominantly display human receptors (Chandrasekaran et al., 2008; Jayaraman et al., 2012; Shinya et al., 2006). On the other hand, human alveolar tissue sections representative of deep lung region have been shown to prominently express avian receptors and are typically stained by HA from avian-adapted influenza A viruses (Chandrasekaran et al., 2008; van Riel et al., 2007). Paraffinized human tracheal and alveolar (US BioChain) tissue sections were deparaffinized, rehydrated, and incubated with 1% BSA in PBS for 30 min to prevent nonspecific binding. HA was precomplexed with primary antibody (mouse anti-6x His tag, Abcam) and secondary antibody (Alexa fluor 488 goat anti-mouse, Invitrogen) in a molar ratio of 4:2:1, respectively, for 20 min on ice. The tissue binding was performed over two different HA concentrations (40 μg/ml and 20 μg/ml) by diluting the precomplexed stock HA in 1% BSA-PBS. Tissue sections were then incubated with the HA-antibody complex for 3 hr at room temperature (RT). The tissue sections were counterstained by propidium iodide (Invitrogen; 1:1000 in TBST). The tissue sections were mounted and then viewed under a confocal microscope (Zeiss LSM 700 laser scanning confocal microscopy). Sialic-acid-specific binding of HAs to tissue sections was confirmed by loss of staining after pretreatment with Sialidase A (from Arthrobacter ureafaciens, Prozyme). This enzyme has been demonstrated to cleave the terminal Neu5Ac from both Neu5Ac2→3Gal and Neu5Ac2→6Gal motifs. In the case of sialidase pretreatment, tissue sections were incubated with 0.2 units of Sialidase A for 3 hr at 37°C prior to incubation with the proteins. Pretreatment of human tissue sections with Sialidase A resulted in complete loss of HA staining.

Binding of HA to Human Tissue Sections

The human tracheal epithelium has been extensively benchmarked as a tissue section representative of the human upper respiratory tract that is a predominant physiological target site for human-adapted influenza A viruses (Imai et al., 2012; Jayaraman et al., 2012; Mansfield, 2007; Shinya et al., 2006). The apical surface and submucosal regions of the human trachea have been shown to predominantly display human receptors (Chandrasekaran et al., 2008; Jayaraman et al., 2012; Shinya et al., 2006). On the other hand, human alveolar tissue sections representative of deep lung region have been shown to predominantly express avian receptors and are typically stained by HA from avian-adapted influenza A viruses (Chandrasekaran et al., 2008; van Riel et al., 2007). Paraffinized human tracheal and alveolar (US BioChain) tissue sections were deparaffinized, rehydrated, and incubated with 1% BSA in PBS for 30 min to prevent nonspecific binding. HA was precomplexed with primary antibody (mouse anti-6x His tag, Abcam) and secondary antibody (Alexa fluor 488 goat anti-mouse, Invitrogen) in a molar ratio of 4:2:1, respectively, for 20 min on ice. The tissue binding was performed over two different HA concentrations (40 μg/ml and 20 μg/ml) by diluting the precomplexed stock HA in 1% BSA-PBS. Tissue sections were then incubated with the HA-antibody complex for 3 hr at room temperature (RT). The tissue sections were counterstained by propidium iodide (Invitrogen; 1:1000 in TBST). The tissue sections were mounted and then viewed under a confocal microscope (Zeiss LSM 700 laser scanning confocal microscopy). Sialic-acid-specific binding of HAs to tissue sections was confirmed by loss of staining after pretreatment with Sialidase A (from Arthrobacter ureafaciens, Prozyme). This enzyme has been demonstrated to cleave the terminal Neu5Ac from both Neu5Ac2→3Gal and Neu5Ac2→6Gal motifs. In the case of sialidase pretreatment, tissue sections were incubated with 0.2 units of Sialidase A for 3 hr at 37°C prior to incubation with the proteins. Pretreatment of human tissue sections with Sialidase A resulted in complete loss of HA staining.

Capturing Network Interamin Acid Contacts for RBS Residues

The coordinates of Neth03 H7 HA-avian receptor (PDB ID: 4DJ7) and Aich68 H3 HA-human receptor complexes (PDB ID: 2YPG) were uploaded into the PDBePISA server (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) to determine key residues in the HA RBS that make contact with the correspond-
2009 H1N1 hemagglutinin increases human receptor affinity and leads to efficient airborne viral transmission in ferrets. PLoS ONE 6, e17616.


Note Added in Proof

Since the submission of this manuscript, a study reporting inefficient aerosol transmissibility of H7N9 in ferrets was published. These data support our predictions and are consistent with the data presented in this study regarding the distinct receptor tropism of H7N9 viruses. Zhu, H., Wang, D., Kelvin, D.J., Li, L., Zheng, Z., Yoon, S.W., Wong, S.S., Farooqui, A., Wang, J., Banner, D., et al. (2013). Infectivity, transmission, and pathology of human H7N9 influenza in ferrets and pigs. Science. Published online May 23, 2013. http://dx.doi.org/10.1126/science.1239844.