VIRAL FACTORS IN INFLUENZA PANDEMIC RISK ASSESSMENT

Marc Lipsitch*, Center for Communicable Disease Dynamics, Departments of Epidemiology and Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, MA, USA

Wendy Barclay, Division of Infectious Disease, Faculty of Medicine, Imperial College, London, UK

Rahul Raman, Department of Biological Engineering, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge MA 02139 USA

Charles J. Russell, Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678, USA

Jessica A. Belser, Centers for Disease Control and Prevention, Atlanta, GA, USA

Sarah Cobey, Department of Ecology and Evolutionary Biology, University of Chicago, Chicago, IL, USA

Peter Kasson, Departments of Biomedical Engineering and Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA, USA

James O. Lloyd-Smith, Department of Ecology and Evolutionary Biology, University of California at Los Angeles, CA, USA and Fogarty International Center, National Institutes of Health, Bethesda, MD, USA

Sebastian Maurer-Stroh, Bioinformatics Institute, Agency for Science Technology and Research, Singapore, Singapore; National Public Health Laboratory, Communicable Diseases Division, Ministry of Health, Singapore, Singapore; School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Steven Riley, MRC Centre for Outbreak Analysis and Modelling, Department of Infectious Disease Epidemiology, School of Public HealthImperial College London

Catherine Beauchemin, Department of Physics, Ryerson Unversity, Toronto, Canada

Trevor Bedford, Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Thomas C Friedrich, Department of Pathobiological Sciences, University of Wisconsin School of Veterinary Medicine, Madison, WI, USA

Andreas Handel, Department of Epidemiology and Biostatistics, College of Public Health, University of Georgia, Athens, GA, USA

Sander Herfst, Department of Viroscience, Erasmus Medical Center, Rotterdam, NL

Pablo R. Murcia, MRC-University of Glasgow Centre for Virus Research, Glasgow, UK

Benjamin Roche, IRD/UPMC UMMISCO, Montpellier, France

Claus O. Wilke, Center for Computational Biology and Bioinformatics, Institute for Cellular and Molecular Biology, and Department of Integrative Biology. The University of Texas at Austin. Austin, TX 78712 Street address: 2500 Speedway, A4800.

Colin Russell, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK

*To whom correspondence should be addressed. Department of Epidemiology, Harvard T.H. Chan School of Public Health, 677 Huntington Avenue, Boston, MA 02115 USA.

mlipsitc@hsph.harvard.edu

ABSTRACT

The threat of an influenza A virus pandemic stems from continual virus spillovers from reservoir species, a tiny fraction of which spark sustained transmission in humans. To date, no pandemic emergence of a new influenza strain has been preceded by detection of a closely related precursor in an animal or human. Nonetheless, influenza surveillance efforts are expanding, prompting a need for tools to assess the pandemic risk posed by a detected virus. The goal would be to use genetic sequence and/or biological assays of viral traits to identify those non-human influenza viruses with the greatest risk of evolving into pandemic threats, and/or to understand drivers of such evolution, to prioritize pandemic prevention or response measures. We describe such efforts, identify progress and ongoing challenges, and discuss three specific traits of influenza viruses (hemagglutinin receptor binding specificity, hemagglutinin pH of activation, and polymerase complex efficiency) that contribute to pandemic risk.

I**NTRODUCTION**

Aquatic birds are the main reservoir of influenza A viruses in nature (1). Influenza viruses from aquatic birds sporadically enter terrestrial bird and mammalian host populations and achieve sustained circulation in these new hosts (2), sometimes after reassortment with influenza viruses already circulating in the new host (3). Adaptation of viruses from aquatic birds to mammals involves a change in tissue tropism from intestinal to respiratory epithelia (4, 5).

Multiple influenza A subtypes—defined by the patterns of antibody recognition of two surface proteins, hemagglutinin (HA) and neuraminidase (NA)—circulate in avian species and swine at any given time. Among these, a number are known to cause sporadic zoonotic infections in humans (6). Hundreds of human infections with avian influenza viruses were detected in the last decade, for example H5N1 and H7N9 (7) as well as swine influenza viruses, e.g. an H3N2 variant that spilled over into humans attending agricultural shows in the early 2010s, H3N2v (8). In addition, zoonotic infections with other viruses from poultry have occurred, including for example H7N7 (9), H10N8 (10), H6N1 (11), H9N2 (12), and H5N6 (13); for more examples and a fuller discussion see (14). The severity of zoonotic influenza A infections ranges from clinically inapparent (15, 16) to fatal (17, 18).

Although secondary transmission can occur following some of these spillover events (19), only a very small proportion of them—four in the last hundred years, which seems to be close to the historical average (20)—led to sustained person-to-person

transmission with global spread (Box 1). There are 18 known HA types and 11 known NA types (21), which could theoretically be found in any combination. So far, sustained spread in humans has been limited to the H1N1, H2N2, and H3N2 subtypes (22), though it is possible that other subtypes circulated prior to 1918, the year of the first pandemic from which viruses are available for study (23). Multiple virus–host interactions are necessary for replication and onward transmission; the differences in the genetic requirements to accomplish each of these interactions in humans versus other animals provide a barrier to sustained transmission following spillover (24).

Experiments in ferrets have been used to measure viral transmissibility via respiratory droplets (in this review we use this term to refer to any transmission through the air between ferrets that are not in direct or indirect physical contact). Droplet transmission in ferrets is a useful, albeit imperfect, correlate of the potential of influenza strains to transmit efficiently in human populations (25). For this reason, some have argued that there is a general phenotype of "transmissibility by respiratory droplets in mammals" such that experiments to select for such transmission in droplets in ferrets are important models of the process of adaptation to human transmission (26, 27). This view is not universally shared (28). Starting from an zoonotic highly pathogenic avian influenza isolate from a human case of infection (or a reassortant of the HA from a different zoonotic H5N1 highly pathogenic avian influenza isolate, with the other segments from the 2009 pandemic H1N1 strain), it was shown that certain specific traits that had been previously associated with mammalian host adaptation were required to achieve respiratory droplet transmission. These ferret-transmission phenotypes in turn

were associated with certain genetic changes relative to the original avian viruses (26, 27, 29). These specific changes occur both in HA and in polymerase-complex proteins.

The rationale for these experiments was that, because the ferret model recapitulates many features of human infection, changes identified in adaptation to ferret transmission would also be important for adaptation to sustained transmission in humans (30, 31), though this can never be known with certainty (28). Notably, viruses isolated from humans who were infected by contact with birds show some of these changes (32, 33), particularly the change at amino acid 627 of the PB2 gene (34-36), which often affects polymerase complex efficiency (see below). This indicates that even the first generation of human infection from nonhuman hosts can initiate a process of host adaptation. It also indicates that not all the human-adaptive changes must be in place in the avian reservoir to initiate this process. Some human infections, including some zoonotic cases (17, 35, 37, 38) and some cases early in a pandemic (39-43), involve viruses that are not yet fully human-adapted (see below and Tables 2–4). The interpretation of some of these isolates is complicated by uncertainty about whether they were passaged in hen's eggs at some point in their history.

Certain types of countermeasures against an influenza pandemic are effective only against one lineage of viruses – for example, creating stockpiles or seed stocks of vaccines against a particular subtype, or culling poultry infected with that subtype. It is not currently feasible to invest in such countermeasures against all viruses circulating in avian or other reservoirs, or even against all those causing known zoonotic cases.

Therefore, there would be value in an accurate system to assess the relative pandemic risks posed by each virus and prioritize them for the development of such strain-specific countermeasures, while directing fewer resources to strains of lower concern (44). This consideration has motivated calls for comprehensive analysis of all available data to assess the threat to public health posed by these strains. One response is the CDC's Influenza Risk Assessment Tool (IRAT) (45), which incorporates elements including properties of the virus, field and epidemiological findings, and attributes of the human population to provide a framework to differentiate among novel influenza viruses thought to possess pandemic potential. Such risk assessments can help focus pandemic prevention and response efforts on the viruses thought to pose the highest risk of pandemic spread (30), in the most worrisome cases providing a rationale for costly measures such as poultry culling or vaccine seed stock development, or even stockpiling of large quantities of vaccine. A guiding question of this article is to examine the degree to which it is justified to rely on measurements and predictions of viral genetic and phenotypic traits in prioritizing responses to particular viral subtypes and within-subtype lineages.

There are several hurdles to evaluating the accuracy of such predictions (24). Factors limiting our ability to identify high-risk viruses and predict the risk they pose include:

- limited surveillance of nonhuman influenza viruses, such that high-risk viruses may not be detected and hence cannot be assessed (46). Limitations include both the

number, geographic and species diversity of hosts sampled, and the difficulty in sampling all genetic variants present in a given infection (47, 48);

- failure to fully characterize some viruses that are detected (49);
- imperfect public health systems lacking capacity to detect zoonotic infections presenting in patients (50, 51);
- epistasis and other complexities that prevent straightforward prediction of viral traits from genotype (24, 52-58);
- technological limitations in molecular modeling and phenotypic assays that limit confidence in predicting and measuring viral traits (59);
- uncertainties about the taxonomic level at which risk predictions should be made (Box 2);
- practical, ethical and cost limitations of animal transmission experiments, as well as some exceptions to the correlation between human transmissibility and droplet transmissibility in nonhuman animal models (25);
- lack of data on immediate animal precursors of viruses that caused previous pandemics;
- multiple scales at which viral strains compete and hence experience selection (i.e. replication within hosts, transmission between hosts). Evolutionary theory for such multi-scale selection is incomplete. Viral fitness components are rarely measured at both scales for the same strain and are imperfectly correlated across scales(60-62);
- the role of stochastic events in the ecology and evolution of influenza viruses during and after host-switching to humans (60, 63), including the potential for

transmission bottlenecks to either promote or inhibit emergence of human-adapted viruses (48, 64-66).

These difficulties are exacerbated by the fact that influenza pandemics are rare events, and that risk assessments are not yet made with enough quantitative precision to formally evaluate their practical application. Even perfect information about the viral determinants of pandemic risk might only be enough to distinguish between strains with a low risk of causing a pandemic (say, 0.1% per year) and those with an extremely low risk (say, less than 0.01% per year), with unpredictable ecological or evolutionary contingencies determining which of these low-probability events will actually occur. One such contingency is that an avian influenza virus could acquire one or more of the determinants of pandemic potential by reassorting with a human seasonal influenza virus.

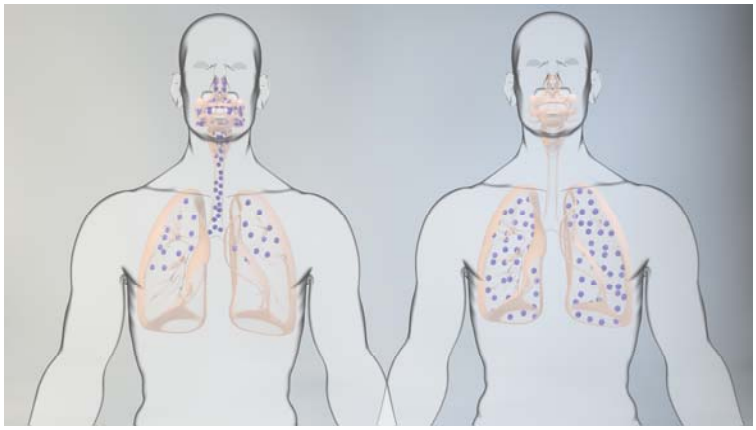
With only one pandemic every few decades, the data set for testing the prediction of such rare events is inadequate, a problem that challenges predictions in many fields beyond infectious diseases (67, 68). Evolutionary events in which a strain increases human-to-human transmissibility, but not enough to spark a pandemic, are extremely hard to observe, but if we could do so it would increase our ability to characterize the process of adaptation (69).

Despite these challenges, there has been tremendous interest and investment in making and using such predictions, and a number of new ideas to improve predictions are in

various stages of development (Box 3). Building on the findings of a previous workshop (24), we considered in detail the present state of knowledge concerning three phenotypic traits: HA receptor binding specificity, and HA pH of activation, and polymerase complex activity, (Figure 1). These were chosen from a longer list of candidate traits (Table 1) because they span the viral life cycle (Figure 1) and their role in host adaptation has been extensively studied. All three are believed to be required for an influenza virus to cause a pandemic; consistent with this assumption, all three traits have been present to some degree in the earliest viruses isolated in pandemics since the 20th century, though some have been enhanced by subsequent evolution during seasonal transmission in humans. Moreover, for each of these three traits, viruses isolated from avian hosts typically do not show the mammalian-adapted phenotype, reflecting divergent selection pressures in the two classes of hosts (Tables 2, 3, and 4). All three traits changed in the adaptation of zoonotic H5 influenza viruses to droplet transmission in ferrets (26, 29). We emphasize that each of these traits is quantitative, and that human-adaptation is not a threshold criterion but a continuum; in this review when we speak of human adaptation we mean a tendency to be better adapted to humans, rather than an absolute yes-or-no property.

This review starts with a summary of our knowledge about the role of each of the three functional traits in conferring pandemic potential on a virus strain. Following these case studies, we draw some generalizations about the prospects of predicting pandemic risk from virus genotype or from assays of particular viral traits. For each trait we present a table showing the degree to which the sequence changes or phenotypic properties

associated with avian or human adaptation are present in isolates from birds and humans, respectively. If the avian traits were always found in avian isolates and human traits always in human isolates, only the shaded cells on the main diagonal would be filled. In such a case, however, it is hard to see how viruses would ever make the jump from birds to humans, since so many traits would have to change simultaneously, and indeed the off-diagonal cells are not empty. Finding avian-adapted traits in viruses isolated from humans most often occurs in zoonotic cases, showing that not all human-adapted traits are required for the first human infection. In some cases there are also viruses isolated from humans after a pandemic starts that retain some degree of avian-like traits, and we discuss these in more detail in the text -- these represent the greatest challenge to use of genotypic or phenotypic information for pandemic prediction because they run the risk of false negatives. The other off-diagonal cell, which represents avian isolates with some human-like traits, simply shows that some circulation of viruses in birds is possible without the classical "avian" phenotypes. How this happens is a phenomenon worthy of further study. We conclude with some recommendations for future research and for the practice of pandemic risk assessment.



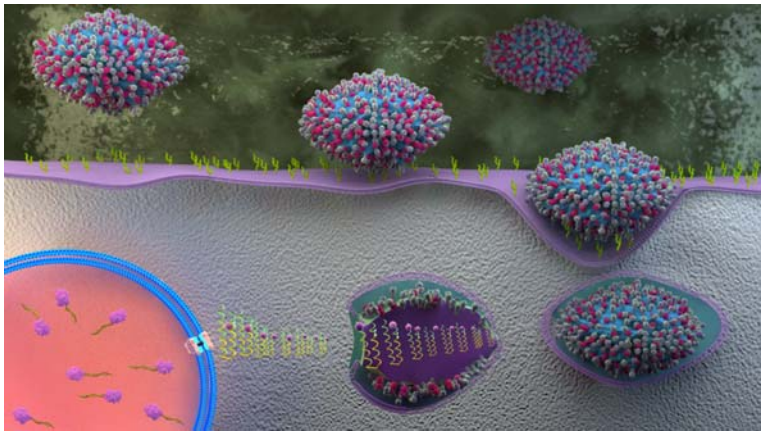


FIGURE 1. Key phenotypic traits for the adaptation of avian influenza viruses to replicate efficiently in humans. A. A switch in receptor binding preference from avian-like (α2,3-linked sialic acid) to human-like (α2,6-linked sialic acid) receptors. The human form on the left shows the typical distribution of human adapted influenza viruses determined by their receptor binding preference for a2,6, linked SA that is predominantly expressed in the upper respiratory tract but also in the lungs. The human form on the right shows that infection with avian influenza viruses is concentrated in the lungs where their preferred a2,3 linked SA receptor is expressed. **B**. Lower HA pH of activation and increased polymerase complex efficiency. Free-floating viruses that enter the human respiratory tract (upper part of figure) encounter mucus and a mildly acidic extracellular environment that act as innate barriers to virus infection. If NA is able to desialylate decoy receptors on mucus and HA has a sufficiently low pH of activation, then the virus particle may reach the apical surface of the respiratory epithelium intact. There through a multiplicity of interactions between HA and cell-surface sialic acid, the virus enters the target cell. After the virus is internalized, it passes through the endosomal pathway where the pH is progressively decreased. The low pH of the endosomal environment triggers an irreversible conformational change in HA that fuses the viral and endosomal membranes and ultimately results in the release of virus genetic material in the form of the viral ribonucleoprotein complex (vRNP) into the cell cytoplasm. The eight vRNPs are subsequently imported into the cell nucleus by interactions between the vRNPs and cellular nuclear import machinery. Inside the nucleus the virus polymerase complex replicates the virus genome in conjunction with co-opted cell proteins.

252

253 **TABLE 1:** Influenza virus adaptations that appear to be required for human-to-human

254 transmission

Trait	**Adaptation**
HA receptor binding specificity	Preference for α2,6-linked mammalian sialic acid receptors over α2,3-linked avian ones (70)
HA pH of activation	HA avoids extracellular inactivation and undergoes conformational changes leading to membrane fusion at appropriate pH for human cells (5.0-5.4 or perhaps 5.5) (59)
Polymerase complex efficiency	Efficient replication in human cells (71, 72)
Virus morphology	Filamentous morphology associated with several adaptations to mammals (73-76)
Length of NA stalk	Longer stalk of NA required to penetrate human mucus and deaggregate virions (77)
Antagonism of interferon production	Species-specific binding of the NS1 protein to host factors (78)
HA-NA "balance"	Substrate selectivity and catalytic rate of NA are calibrated to "balance" avidity of HA for the cell-surface glycan receptor (79-82)

Trait 1: Hemagglutinin receptor binding specificity

A. Definition of the trait

Attachment of an influenza virus to a host cell requires binding of the viral HA to a sialylated glycan receptor (sialic acid) on the surface of the host cell. Cells of the avian gut and a minority of cells in deep lung in mammals predominantly express receptors terminated with an α2,3-linked sialic acid: hereafter, α2,3 glycans or *avian receptors* (70, 83-85). By contrast, in humans and other mammals, upper respiratory epithelial cells express mainly glycan receptors terminated by α2,6-linked sialic acid: α2,6 glycans or *human receptors* (41, 85, 86). The human upper respiratory epithelium is the primary target site for infection of human-adapted viruses, and infection at this site is thought to be a prerequisite for efficient human-to-human transmission via respiratory droplets. Thus, it appears that human adaptation of an HA is associated with a switch in its binding preference from avian to human receptors. Receptor binding is not either-or; human-adapted influenza virus HA may show some binding to avian receptors, and vice versa.

Receptor binding preference is defined as the ratio of affinity (or avidity) of an HA molecule for an α2,6 glycan relative to that for an α2,3 glycan, with higher values associated with greater human adaptation. The evolution of receptor binding specificity is driven by the host environment, with selection for specificity during the infection process within a host and during the process of transmission. The error-prone replication of influenza genomes can facilitate rapid emergence of viruses with amino

acid substitutions that alter the receptor binding characteristics of the HA (87). Increased transmissibility may result from mammalian receptor adaptation, either because the virus shedding form the infected donor host is increased, or because the ability of virus to infect the recipient host at a low dose is enhanced, or for both of these reasons. Recent experimental evidence in ferrets implicates the soft palate as an important site of selection for α2,6 specificity (87).

B. Genetic and structural determinants of hemagglutinin-receptor interactions

Preference for binding human or avian glycan receptors is determined by the structure of the viral HA. Except for a few conserved amino acids in the sialic acid receptor binding pocket, the influenza HA has considerable structural plasticity to evolve variation at the rim of the pocket to engage different sialic acid linkages. Importantly, antigenic regions of the HA are located nearby regions that determine receptor-binding preference, meaning that selection for antigenic escape may be constrained by the need to maintain receptor preference (88). More speculatively, selection for changes in receptor preference might also alter recognition of the HA by host antibodies.

Conformation of the hemagglutinin as a determinant of receptor-binding preference. Although the co-crystal structures of HA and sialylated glycans have not been solved for all pairs, there is evidence that avian- or human-adapted HA bind to different conformations of the avian and human receptors: the *cis* conformation of human

296 receptors and the *trans* conformation of avian receptors (41, 89-97). This finding has led
297 to the concept of "hallmark" residues within the receptor-binding site of avian- and
298 human-adapted HAs. Avian-adapted HAs typically carry Glu at position 190, Gln at
299 position 226, and Gly and position 228 (H3 numbering), and the Gln226->Leu, Gly228-
300 >Ser substitutions have been associated with a switch to human receptor preference in
301 HAs of H2, H3 (98), and H5 (99) viruses. In H1 HA, Glu190→Asp and Gly225→Asp have
302 been considered as hallmark amino acid changes to switch receptor specificity leading
303 to greater human adaptation (42, 100). The determinants of specificity are reviewed in
304 much more detail in (101).

305 *Additional structural features involved in receptor binding preference.* The *cis* and *trans*
306 definition of glycan conformation does not fully describe HA binding to a range of
307 structurally diverse glycans displayed on human respiratory cells and tissues (86). This
308 limitation motivated studies that revisited the definition of glycan conformation,
309 extending the conformational analysis beyond the terminal sialic acid linkage to
310 describe overall topology and dynamics of the glycan receptor upon binding to the
311 receptor-binding site of avian and human-adapted HAs (86, 102). HA sequence
312 determinants of preference for the "cone"-like topology of avian receptors, versus the
313 "umbrella"-like topology of human receptors, are still being defined (56).

314

315 **C. Experimental assays to measure hemagglutinin receptor binding specificity**

316 Experimental evidence on differential binding of avian and human viruses to sialic acid
317 receptors in avian and human conformations, respectively, was first obtained by
318 hemagglutination assays with erythrocytes whose surfaces had been chemically
319 modified to display glycans terminating with either homogeneous α2,3 or homogeneous
320 α2,6-linked sialic acids (103). Subsequent analysis of the repertoire of glycan structures
321 in erythrocytes of various animal species informed the use of cells from different species
322 as probes of HA receptor binding preference in hemagglutination assays (104).

323 Greater precision and reproducibility has been achieved with the use of purified
324 sialylated glycans to create solid-phase binding assays with fluorogenic or enzymatic
325 detection (105, 106). With these assays, it is possible to characterize the relative direct
326 binding of whole virions or recombinant trimeric HA oligomers to glycans attached to a
327 solid phase or the competition of such glycans with binding to a generic glycoprotein
328 attached to the solid phase (106). In recent work, biosensor interferometry and
329 thermophoresis have been used to measure glycan-binding avidities and affinities in a
330 more precise manner and to relate the two (107).The development of glycan
331 microarrays represented a turning point in the analysis of influenza virus receptor
332 binding specificity, because it allowed simultaneous evaluation of virion or recombinant
333 HA binding to a large repertoire of sialoglycans (41, 108, 109). Several measures of
334 preference for an HA molecule or whole virus have been defined, including the ratio of
335 the number of α2,6 to α2,3 glycans bound (41, 110) or the corresponding ratio of
336 binding affinity or avidity (26, 107). A limitation to predictive power is that glycans
337 tested on current arrays may not match those present in the human respiratory tract

(111). These arrays may also not present glycans in the same fashion as respiratory epithelial cells, so strategies such as measuring the binding of labelled viruses to human respiratory tissues (99) or explant cultures (112) may be promising alternatives, although challenges remain in standardization and quantification of such assays. Structural studies of wild-type and mutant HA in complex with representative sialoglycans provide the ultimate level of detail by characterizing interactions at the atomic level. X-ray crystallography advances in recent years have accelerated structural determination, and similar progress in recombinant protein purification techniques combined with robotic crystal screening have reduced the amount of protein and labor required.

In summary, genetic and protein sequence analysis, glycan arrays, and X-ray crystallography studies provide complementary data towards understanding the sialoglycan interactions of emerging viruses, with tradeoffs of equipment and reagent costs and throughput against level of precision and detail provided.

D. Receptor binding preference as a predictor of host adaptation of influenza viruses and pandemic risk

At present, estimating the contribution of receptor specificity to the pandemic risk posed by a novel virus relies primarily on the similarity between the receptor binding characteristics of the emerging virus and that of the most closely related HA with known transmissibility among humans or a surrogate animal model.

As noted above, hallmark residues have substantial predictive power. These distinct sets of hallmark residues in the H1, H2 and H3 subtype (101) correlate with human-adaptation in known sequences collected from birds or humans (40, 101); they induce changes in receptor-binding specificity when introduced experimentally (113, 114); and experimental selection for receptor binding *in vitro* (113) or in ferrets (26) cause these changes to appear.

However, hallmark residue predictions of receptor-binding specificity are imperfect, as evidenced by a failure to switch *in vitro* receptor-binding preference from avian to human when changes observed in H5N1 strains after selection in ferret gain-of-function experiments were introduced to other H5N1 viruses (57). The involvement of other features in human adaptation, such as the topology of the bound HA-receptor complex, further complicate the genetic prediction of human adaptation, as the residues involved in these features are less well characterized (115).

In principle, phenotypic assays that directly measure the receptor-binding preference of HA – if performed under realistic conditions that capture the interaction of the HA trimer with the receptor (83, 116, 117)—may better capture the trait of interest than genetic predictions of this preference. However, even here, a simple equivalence between binding preference for α2,6-linked glycans and pandemic risk could be misleading. Several viruses circulating in humans during the early phase of previous pandemics were found to show either a preference for avian receptors (39, 40) or a mixed preference for both human and avian receptors (39, 42, 109). In the case of early

2009 pandemic viruses, findings are mixed (109, 118). Some of the findings of dual or avian specificity may reflect artifacts introduced when human isolates were passaged in eggs before receptor specificity was assayed; alternatively, they may genuinely reflect a transitional stage in the evolution of HA genes in human populations after transmission from other species (40-42, 119), (Table 2). Consistent with this latter possibility, an H5N1 virus isolated from a human zoonotic case in Vietnam displayed strong avian receptor preference (120). This preference changed in the course of experiments to adapt it to respiratory droplet transmission in ferrets (26). Taken together, these findings confirm that there is a strong correlation between measured receptor preference and the host from which a virus is isolated. However, they raise questions about the predictive value of human receptor binding preference. Indeed, the examples of mixed receptor preference in human isolates from the early phase of the H1 or H2 pandemics suggest that the ability to evolve human receptor specificity over a chain of human infections, which may be present in many avian-receptor-adapted viruses, may be sufficient for pandemic emergence.

In summary, detection of a human receptor preference in a spillover virus may be an indication of increased risk, but exclusive human receptor preference is probably not necessary for an influenza A virus to initiate a pandemic. With several possible exceptions noted above, most viruses isolated to date fall within the shaded cells in Table 2, which indicates concordance between the source of the isolate and the virus trait. Thus, prioritizing pandemic countermeasures against virus lineages with inferred or measured human receptor preference will likely lead to better targeting of such

countermeasures on average – that is, increase the chance of taking countermeasures against a strain that truly poses pandemic risk. However, the counterexamples of human-to-human transmission of incompletely adapted viruses (bottom left and middle cells of Table 2) suggest that in particular cases, reliance on this trait as a necessary condition to justify countermeasures may not identify all virus lineages that are in fact capable of causing a pandemic.

TABLE 2: Hemagglutinin receptor binding preference and examples of viruses isolated from avian and human hosts showing preference for human or avian receptors, or mixed preference

	Avian receptor preference	**Mixed receptor preference**	**Human receptor preference**
Expected sequence, trait. Hallmark residues HA 190, 225 (H1,H3), 226 (H3); many others	Preferential binding to α2,3 sialylated glycans. HA 190Glu, 225Gly, 226Gln	Similar binding to both classes of glycans	Preferential binding to α2,6 sialylated glycans. HA 190Asp, 225Glu, 226Leu
Found in avian isolates	Many examples: many avian isolates of subtypes H5N1 (32, 120), H2 (40) and H3 (40)	avian isolates of H5N5 (121), North American H7 (122), H7N9 (123), as well as examples from H2 (40, 92) and H3 (40)	Some H9N2 avian isolates (124, 125)
Found in human isolates	H5N1 zoonotic isolate (26, 120); one H1N1 isolate from 1957 (39)*; some early H2N2 pandemic/seasonal isolates (40, 43, 98)*	Early H1N1 pandemic isolates from 2009(109) and 1918 (41, 42); several H1N1 from the 1918-1956 period (39)*; early H2N2 isolate from 1958 (43); human isolate of zoonotic H7N9 (126)	Many examples: H1N1 post-1977 (39); early H1N1 pandemic isolates from 2009 (118) and 1918 (41, 42); most human H2 and H3 seasonal isolates (40, 98)

*These anomalous results are speculated by the authors to be possibly, or even probably the result of laboratory adaptation to egg passage and may not reflect the properties of the primary isolate. A possible counter to this interpretation is that it is seen only in the earliest isolates from human pandemic viruses, while nearly all isolates from after the pandemic year, which should also have been passaged in eggs, show human-adapted phenotypes.

Trait 2: Hemagglutinin pH of activation

A. Definition of the trait

After entry into the cell, influenza viruses are internalized into endosomes, where the pH is progressively decreased. The pH of early and late endosomes, as well as lysosomes, varies between cell types, tissues, and host species. The HA must undergo a low-pH triggered conformational change to a state capable of fusing the viral and endosomal membranes. For human-adapted viruses, HA activation typically occurs between pH 5.0 to 5.5. HA variants that undergo this transition at a higher pH, as is typical for avian influenza isolates, are poorly adapted to infect human cells because the transition can happen prematurely, leading to extracellular inactivation in the mildly acidic mammalian respiratory tract (127, 128) The pH of activation can be defined as a continuous measurement representing the least acidic (highest) pH at which a particular HA molecule is triggered. Greater acid stability (lower pH of activation) is associated with greater human adaptation.

B. Functional, structural and genetic determinants of hemagglutinin pH of activation and its consequences

The HA is synthesized and folded such that the fusion peptide is buried and inactive until specific activation signals are provided. The structural changes that expose the fusion peptide and lead to fusion have been described in detail (129). If the virion is exposed to

437 sufficiently low pH outside of a host or host cell, the HA protein undergoes irreversible
438 structural changes too early and is unable to mediate virus entry; such virions become
439 inactivated. Thus the term *acid stability* is more broadly used to define the threshold for
440 acidification that triggers membrane fusion (in the endosome) or inactivation (if
441 triggered outside of the cell for an HA that is not sufficiently stable). During endocytosis,
442 an influenza virion is exposed to sequentially lower pH values in early endosomes (pH
443 6.0-6.5), late endosomes (pH 5.0-5.5), and lysosomes (pH 4.6-5.0) (130). If the HA is too
444 stable, and fusion is not triggered in the acidic endosome of the host cell, further traffic
445 into lysosomes results in virus inactivation by lysosomal proteases (129).

446 Based on surveillance studies, human-transmissible influenza isolates appear to have HA
447 proteins that are more acid stable (have a lower activation pH) than avian influenza
448 viruses (59). The HA activation pH values for H1N1, H2N2, and H3N2 seasonal viruses
449 during the 20th Century range from pH 5.0 to 5.4 (131). In 2009, emerging pandemic
450 H1N1 viruses had HA activation pH values of approximately 5.5, but numerous
451 subsequent isolates have acquired mutations that lower the activation pH to the range
452 of the 20th Century human influenza viruses (132-134). Broad surveys of avian and swine
453 influenza isolates have shown that HA activation pH can vary substantially with a range
454 from pH 4.6-6.0 (131, 135). Among avian viruses, low-pathogenic duck viruses appear to
455 range in acid stability from pH 5.3-6.0 and highly pathogenic avian viruses range from
456 5.6-6.0 (131).

457 Consistent with observed patterns in natural isolates, some experimental evidence
458 indicates that within the range of natural variation, lower activation pH is adaptive for
459 mammalian replication while higher activation pH is adaptive for replication in avian
460 hosts. For isolates of H5N1 highly pathogenic avian influenza virus, an increase in HA
461 activation pH within the range of 5.2-6.0 has been associated with increased replication
462 and pathogenicity in chickens (136). Conversely, a mutation that decreased the HA
463 activation pH of A/chicken/Vietnam/C58/2004 (H5N1) from 5.9 to 5.4 has been shown
464 to attenuate virus growth and prevent transmission in mallard ducks (137) but increase
465 virus growth in the upper respiratory tracts of mice and ferrets (128, 138). Therefore,
466 for H5N1 viruses, a higher HA activation pH (5.6-6.0) has been associated with a
467 component of fitness in birds, and a lower HA activation pH (pH 5.0-5.4) has been linked
468 to greater replication in the mammalian upper respiratory tract. Two H5N1 viruses were
469 adapted to transmit by the airborne route between ferrets (26, 27). After a switch in
470 receptor-binding specificity from avian to human receptors (as described above) and
471 deletion of a glycosylation site, in both studies a final mutation that decreased the HA
472 activation pH was shown to be necessary for airborne transmissibility in ferrets.
473 However, these and other studies have shown that this acid stability change is not
474 sufficient in the absence of human receptor-binding specificity (128, 139). Recently, an
475 HA protein whose activation pH was 5.5 or lower was shown to be required for the
476 pandemic potential of 2009 pH1N1 influenza virus (134).

477 Nearly 100 mutations have been described to alter the HA activation pH values of
478 various influenza A virus subtypes (59, 140). These acid stabilizing/destabilizing residues

are located throughout the HA1 and HA2 subunits and tend to be positioned in regions of the molecule that undergo large-scale changes in structure during pH-activated protein refolding (59, 141, 142). Mutations that modify the activation pH do not appear to alter the prefusion HA protein backbone in X-ray crystal structures (136, 143, 144). Therefore, an experimental determination or modeling of intermediate structures may be required in order to reliably predict HA pH of activation. Further complicating genetic prediction of HA activation pH values are observations that the NA and M proteins can also modulate HA acid stability in some cases (145-148).

C. Experimental assays to measure hemagglutinin activation pH

A variety of experimental techniques have been developed to measure the activation pH of the HA protein, quantified as the highest pH at which the HA protein is activated to undergo the irreversible structural changes that mediate membrane fusion (149), or alternatively the highest pH at which, in the absence of a membrane with which to fuse, the HA protein is inactivated (inactivation pH). Classical membrane fusion assays have measured the property in bulk (150). The pH of inactivation can be measured using aliquots of virions that are exposed to buffers of progressively lower pH and, after restoration to neutral pH, assayed for retention or loss of infectivity (135). In many classical fusion assays, fluorescent probes are used to label virions, HA-expressing cells, and/or target liposomes or cells. In these *in vitro* assays, HA-bound target cells are typically exposed to buffers of various pH values and then lipid and/or contents mixing are measured by fluorescence (151, 152). Alternatively, cell monolayers expressing

cleaved HA proteins can be pulsed by low-pH buffers and then incubated to readout HA-mediated cell-to-cell fusion either microscopically by syncytia formation or by reporter gene expression. If HA conformation-specific monoclonal antibodies are available for the subtype being studied, HA-expressing cells can be pulsed with low pH and then analyzed for conformational changes by flow cytometry (137). If such antibodies are lacking, HA-expressing cells can be assayed for trypsin susceptibility after low-pH exposure, with prefusion HA being resistant and postfusion HA susceptible to trypsin degradation (153). Recently, methods have been developed to study HA activation and membrane fusion by individual virions, including single virion fusion using total internal reflection fluorescence microscopy (149).

Although the biological trigger for HA's conformational change is a drop in pH, HA refolding can also be triggered by other destabilizing agents such as heat and urea (135, 154, 155). Stability at a lower pH is associated with stability at higher temperatures and higher urea concentrations, permitting the use of these agents instead of, or in addition to, pH in assays of stability. Thermal stability has been determined by measuring the threshold temperature at which denatured HA protein loses its ability to bind erythrocytes and cause hemagglutination (29).

D. Role of hemagglutinin activation pH in pandemic risk prediction

Many questions remain regarding whether HA activation pH plays a similar role in all influenza subtypes isolated from a wide variety of avian species. For early isolates of the H1N1pdm lineage in 2009, the HA protein has an activation pH of 5.5, which appears

521 intermediate between the canonical human (lower) and avian (higher) ranges.
522 Subsequent H1N1pdm isolates have HA activation pH values ranging from 5.2-5.4,
523 suggesting pH 5.5 may be the upper limit for human pandemic potential and a lower
524 value may be preferred. Indeed, a destabilizing HA mutation in the background of
525 H1N1pdm results in a loss-of-function of airborne transmissibility in ferrets and has
526 been reported to be followed by re-gain-of-function by mutations that lower the HA
527 activation pH to 5.3, a value representative of human-adapted H1N1pdm viruses (134).
528 For the moment, it appears that while HA pH of activation that is shown experimentally
529 to be suitable for human infection is highly typical of isolates from human pandemic and
530 seasonal influenza (Table 3, bottom right) (131), it is possible for humans to have
531 symptomatic infection with (though not extensively transmit) viruses with activation pH
532 closer to the range associated with terrestrial birds (Table 3, bottom left). Conversely
533 (Table 3, top right), there are avian H9, H10, H14, and H15 isolates that display
534 activation pH typical of human viruses (131). The existence of these human-like avian
535 viruses is perhaps unsurprising, as they may lack other essential adaptations for human
536 transmission. As in the case of receptor binding, reliance on this trait to prioritize
537 pandemic prevention measures should consider this property in conjunction with other
538 properties associated with pandemic potential and will likely enrich the coverage of
539 truly high-risk strains on average.

540 Systematic assessment of the predictive value of HA activation pH will require broad
541 empirical testing, since nearly 100 residues throughout the HA molecule have been
542 implicated in regulating HA pH of activation. Predicting activation pH from sequence will

543 therefore require more extensive data. To address this issue, sequencing studies
544 combined with phenotypic assays could be performed on a large range of HA variants to
545 determine the effects of pH-altering mutations in different HA subtypes. High-resolution
546 determination of HA structural intermediates may assist in developing molecular
547 modeling approaches to calculate HA stability from sequence. In the interim, there is a
548 pressing need to develop high-throughput assays for HA pH of activation, along with
549 other properties believed important to interspecies adaptation, in the thousands of
550 surveillance samples obtained annually.

551

552

553 **TABLE 3:** Hemagglutinin pH of acivation

	Avian-adapted for transmissibility	Human-adapted for transmissibility
Expected trait	pH of fusion >5.4 (147)	pH of fusion 5.0-5.4 (5.5 for early H1N1pdm) (134)
Found in avian isolates	Avian H1-H4, H11 isolates (131, 134, 136, 147)	Avian H5, H8, H9,H10,H14,H15 isolates (131)
Found in human isolates	H5N1(26, 29) and H7N9 (123) human zoonotic isolates with pH ≥5.6. One human H1N1 (2008) isolate.	Human isolates of H1N1 (1918 and 2009 lineages), H2N2, H3N2 (131)

554

555

Trait 3: Polymerase complex efficiency

A. Definition of the trait

The heterotrimer of influenza polymerase subunits (PA, PB1, PB2 gene products, together forming the RNA-dependent RNA polymerase) and the nucleoprotein (NP gene product) is required to transcribe and replicate the viral genome (156). The polymerase genes of viruses isolated from avian hosts show a number of genetic differences from their counterparts in viruses isolated from humans (37), and avian virus polymerase typically performs inefficiently in replicating the viral genome in human cells (71, 72). Adaptation to efficient human-to-human transmission requires efficient activity of this complex of proteins, which we refer to as the polymerase complex, in human cells (71, 72).

B. Genetic basis of polymerase complex efficiency.

Some mutations in PB2 are consistently associated with efficient function of the polymerase complex in mammalian cells (Figure 2). As long ago as 1977, it was shown that an avian influenza virus could achieve efficient replication in mammalian cells by acquiring mutations solely in the PB2 subunit of the viral polymerase (157). The most famous of these mutations was later described as PB2 residue 627 (158), which is a glutamic acid (Glu) in avian influenza viruses but a lysine (Lys) in human-adapted viruses, including those that emerged in the pandemics of 1918, 1957 and 1968, and their seasonal descendants. An important exception is the virus that sparked the pandemic

of 2009. In this virus, the PB2 segment had been introduced from an avian precursor into swine viruses in the 1990s, and mammalian adaptation had been achieved by a different set of PB2 mutations including changes at residues at 271, 590 and 591 (159). Now that the 3-dimensional structure of the viral polymerase has been elucidated, we can see that residue 627, 271, 590 and 591 lie on the same external surface. Mammalian-adapting mutations increase the positive charge of this domain, suggesting that they either adapt the virus for interaction with an enhancing host factor or enhance its ability to repel a restriction factor (159). Recently a host factor, ANP32A, that differs between mammals and flighted birds was shown to be a cofactor of the influenza polymerase, and the species specific difference could explain the inefficient function of avian virus polymerase and the stringent selection for the 627Glu->Lys adaptive mutation in mammals (160).

Another residue implicated in mammalian adaptation of the polymerase is residue 701 of PB2, which lies close to but is distinct from the 627 cluster. It has been suggested that this mutation and others in this domain at residues 702 and 714 affect the interaction between PB2 and importin-alpha isoforms either in a way that enhances nuclear import of newly synthesized PB2 or that affects polymerase function once inside the nucleus, the site of viral RNA replication (161-163). Other mutations have been described that adapt PB2 for the mammalian nucleus (for example the triplet threonines at positions 147, 339 and 588) but whether they affect interaction with ANP32A, importins or as yet unidentified host factors is not yet elucidated.

The adaptive value of these mutations is shown by experimental or observational data in which a mammalian host is infected with a virus whose PB2 is not adapted for efficient mammalian replication, but such a mutation becomes common in the virus population over the course of infection. Such evolution has been observed in a fatal human case of influenza A/H7N7 (34) and in mouse experiments following serial lung passage using an isolate from this outbreak (164). Lys at position 627 has also been associated with greater severity in zoonotic H7N9 (38) and H5N1 (17) cases However, reverse genetics experiments show that certain strains of avian influenza may be less able to accept these mutations than others (165).

C. Experimental assays to measure polymerase complex efficiency in human cells

Polymerase complex efficiency in human cells can be measured by an *in situ* assay in which the influenza polymerase is reconstituted from cloned cDNAs in plasmids and then coexpressed with "minigenome," a viral-like RNA encoding a reporter, such as luciferase. By measuring the rate of reporter accumulation in the transfected human cell line, specific combinations of RNA sequences for the polymerase-complex viral genes can thereby be screened directly for their efficiency in producing the mRNA encoding the reporter gene product, providing a measure of human adaptation of the polymerase complex (166).

The original form of the *in situ* reconstituted polymerase assay requires expression of just the minimal set of four viral proteins to replicate the minigenome RNA: PB1, PB2, PA and NP. However, recent work showed an important additional role for another

618 protein, the nuclear export protein (NEP), which is translated from a spliced mRNA
619 derived from RNA segment 8 (that also encodes the major interferon antagonist NS2)
620 (167). In human H5N1 isolates that do not contain PB2 host-adapting mutations, the
621 inefficient activity of these avian polymerases in human cells could also be compensated
622 for by certain mutations in NEP (168). It appears that NEP is an important regulator of
623 the balance between transcription and replication (169, 170), and can thus enhance
624 fitness in viruses containing otherwise inefficient polymerases. The mechanism of this is
625 as follows: the polymerase-enhancing domain of NEP is masked when NEP is folded in
626 one conformation. However, mutations that increase the ability of NEP to rescue avian
627 polymerase function allow more ready unfolding of the protein, unmasking the
628 "activating" domain at the lower temperature of the mammalian respiratory tract.
629 Interestingly, NEP overexpression in cells in which human-adapted polymerase is
630 reconstituted is inhibitory because excess complementary RNA accumulates at the
631 expense of messenger RNA and further viral RNA replication (171). Thus although a
632 short-term adaptation of avian virus polymerase to mammalian cells can be achieved in
633 this way, it may be that further compensatory changes rebalance NEP function in the
634 face of polymerase adaptation during continued circulation in humans, although direct
635 evidence for this selection is lacking. Indeed, although the rescue of low polymerase
636 activity by NEP may explain the human infections by H5N1 viruses that lack other
637 polymerase adaptations, it is not clear that such rescue is sufficient to create a level of
638 transmissibility consistent with pandemic spread. Nonetheless, this finding shows that
639 the minimal polymerase assay is not always sufficient to predict viruses that have

functionally adapted polymerase activity to human cells and that a role for other viral proteins including at least NEP should also be considered in assessment of polymerase function.

Alternatively, polymerase activity could be measured in the context of viral infections (although this will require proper containment). This could be achieved by measuring intracellular levels of viral transcripts using transcriptomics or qRT-PCR. Such experiments would provide important information if they are performed using appropriate cell lines (or respiratory explants) at the temperature of the human airway (33^{o}C).). It has been suggested that plaque size at 33^{o}C can be used as a surrogate measure of polymerase function but plaque size is a mutligenic trait. The predictive value of such assays for transmissibility is limited.

Ultimately, it would be valuable to develop a simple screen to assess the ability of a viral polymerase to support replication and transmission in humans. This phenotype is influenced by at least 4 different viral genes and involves interactions with several different human host factors. If all the relevant host factors were enumerated, one could imagine quickly converting sequence information into an assay that tested for interactions that should support activity. Along these lines the recent description of a host factor, ANP32A that differs between flighted birds and mammals and explains the poor activity of avian polymerase in mammalian cells is a step forward (160).

D. Role of polymerase complex efficiency in pandemic risk prediction

660 The inefficient polymerase of avian influenza viruses in mammalian cells is one of the
661 host-range barriers that likely diminishes pandemic risk. Unlike the requirement for
662 adaptive mutation in the novel HA, this polymerase barrier can be rather readily
663 overcome by reassortment in which an avian virus with novel antigenicity can acquire
664 one or more polymerase genes from mammalian-adapted viruses. In addition,
665 adaptation of avian virus polymerase by accumulation of adaptive mutations in either
666 the polymerase genes or possibly in other viral genes such as the NS segment encoding
667 NEP can enhance avian virus polymerase function sufficiently to support a host range
668 jump.

669 Many H5N1 viruses that circulate today in the avian reservoir already have mutations in
670 PB2 at 627 (165) or 701 (17), likely resulting from the reintroduction of mammalian-
671 adapted strains back into the wild bird reservoir. These have been associated in human
672 infections with more severe cases (17). The fact that these strains have not achieved
673 sustained human-to-human transmission demonstrates that while polymerase
674 adaptations to humans are likely necessary, they are not sufficient for a strain to spark a
675 pandemic. Moreover, the absence of the signature PB2 627K mutation in the 2009 H1N1
676 pandemic strain demonstrates the limitations of relying on any single mutation for risk
677 prediction (172); viruses with the avian-like residue have also been isolated from
678 zoonotic human cases of H5N1, H7N9, and H9N2 infections (Table 4, bottom left). On
679 the other hand, the concept that adaptation of the polymerase is necessary for
680 sustained human transmission is validated by findings that the 2009 pandemic strain
681 had adapted to replication in human cells by changes other than E627K within the

682 polymerase (159). Identification of biophysical mechanisms common to mammalian-
683 adaptive mutations may in the future provide the basis for new biological or biophysical
684 assays of polymerase function to inform risk predictions.

685 In summary, no single polymerase mutation appears to be predictive of pandemic risk
686 for all viruses, but the concept that the polymerase must adapt to human cells before it
687 can cause extensive human-to-human transmission appears consistent with the four
688 pandemic jumps that have occurred in modern times.

689

690 **TABLE 4:** Polymerase complex efficiency; entries list amino acid at PB2 627, though

691 other residues are clearly relevant to this trait.

	Avian-adapted	Human-adapted
Expected trait	Low efficiency in mammals, PB2 590/591 G/Q, 627E, 701D	High efficiency in mammals, PB2 590/591 S/R, PB2 627K, 701N;
Found in avian isolates	Nearly all avian sequences in databases as of 2005 (37)	A few entries in databases show sequences associated with human adaptation as of 2005 (37)***
Found in human isolates	zoonotic H9N2 (37); some zoonotic H5N1 (17, 37); some zoonotic H7N9 (associated with milder course) (38); one zoonotic H5N6 (173)**	Pandemic and seasonal H1N1, H2N2, H3N2 from 1918-2008 (71) ; some zoonotic H5N1 (37); some zoonotic H7N9 (associated with more severe course) (38); H1N1pdm (172)* ; one zoonotic H5N6 and one zoonotic H10N8 (173)

692 *the role of amino acids 590 and 591 in adaptation was not recognized until after the

693 2009 strain had already emerged (159); it has the residues associated with avian

694 adaptation at sites 627 and 701 that were known at that time (172).

695 ** complete sequence information not given in the paper

696 *** the rarity of these raises questions about possible sequencing errors.

697

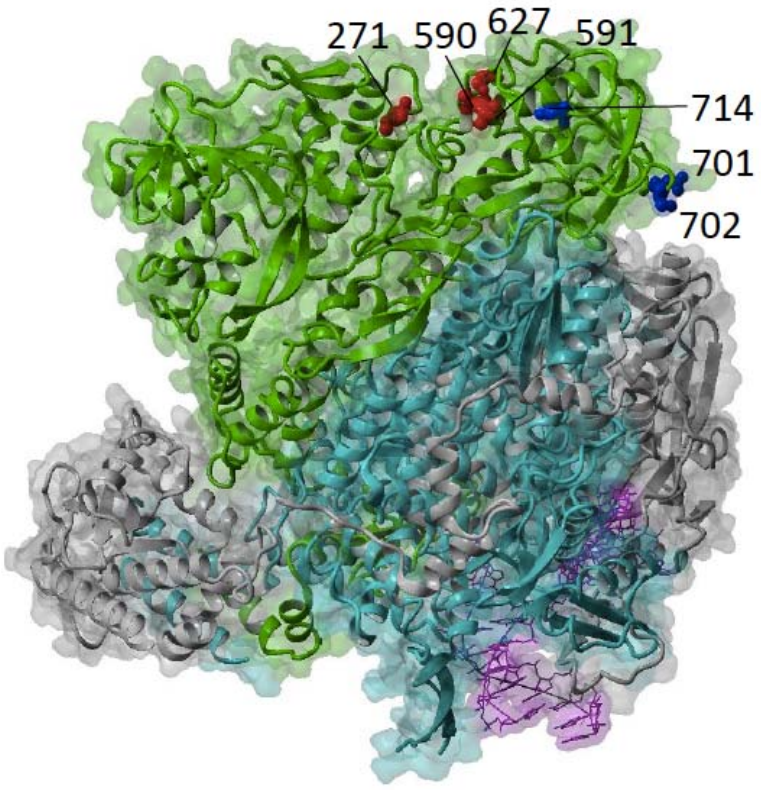


FIGURE 2: Influenza A polymerase complex from structure PDB:4WSB (174) consisting of PA (grey), PB1 (cyan), PB2 (green) and bound vRNA promoter (purple). Key host adaptation sites are indicated as red balls. Sites for importin-alpha interaction are shown as blue balls. Structure visualized with YASARA (175).

DISCUSSION

There has been tremendous progress in understanding the traits involved in the adaptation of avian influenza viruses for efficient human-to-human transmission and the genetic and structural basis of each of these traits. While the ability to use virus sequence data to inform risk assessment of pandemic potential is improving, it remains essential to consider these data alongside other experimental and epidemiological data. For example, in 2013 there was a substantial increase in the number of human infections with A/H5N1 viruses in Cambodia. The increase in infections was cause for substantial concern by itself. Enhancing the level of concern was the finding that some of the viruses collected from infected humans contained previously identified genetic mutations suggestive of human adaptation (30). These findings led to extensive epidemiological and experimental investigations and then to the decision to produce a candidate vaccine virus from a virus representative of the 2013 Cambodian outbreaks.

While predictions of virus phenotypes from sequence data can be informative, they are not infallible, for several reasons, notably the large number of sites involved in determining such traits(56, 59, 71, 159, 168, 172), the important role of epistasis (dependence of a mutation's effect on the genetic background in which it appears) in determining these traits (24, 52-58), and the consequent imperfections in our ability to map single sequence polymorphisms to a trait value. For example, the hallmark HA

amino acid residues 190, 226 and 228 are important to human adaptation, but "human-adapting" mutations at these residues do not always change receptor-binding specificity; it depends on the genetic background. Similarly, amino acid residues 627 and 701 of the PB2 protein are often involved in human adaptation, but both carried the "avian-adapted" residue in the 2009 H1N1 pandemic strain. When these changes were introduced individually or together in the laboratory, the resulting polymerase showed greater activity in a minigenome assay, but replication was unchanged or attenuated in vitro, in mice, and in ferrets (172, 176). After the pandemic strain was identified and its anomalous residues at these sites noted, other sites within PB2 were identified and found to be responsible for human adaptation (159, 177).

Based on the evidence to date, it seems clear that all three of the traits considered in this review, and possibly others in Table 1, must be simultaneously present at least to some degree for a strain to cause a pandemic. Yet with only a few instances of pandemic strains emerging per century, it should not be surprising that a new pandemic strain would violate an apparent rule of human adaptation that applied perfectly to previous pandemic strains, as in the case of the PB2 residues associated with human adaptation in 2009, or as in the case of activation pH of HA in early 2009 isolates (134), which had a value outside the range previously seen in human influenza viruses. It is unclear whether the list of sites and phenotypic traits associated with human adaptation is nearly complete or will continue to grow as we experience additional pandemics.. At least for the traits of receptor binding (109) (Table 2) and acid stability(134) (Table 3), full human adaptation may not be required to initiate a pandemic in a virus that is

748 otherwise well-adapted for humans. Thus, whether or not the list of traits required for
749 pandemic is now complete, our understanding of where the threshold lies for being
750 sufficiently human-adapted continues to change.

751 There are three complementary approaches to address these limitations: improving
752 genetic prediction of biological traits, improving assays of these traits, and improving
753 animal models of human transmission; all approaches are currently progressing in
754 parallel (178, 179). The first approach aims to further refine our understanding of
755 sequence-to-trait relationships by continued studies of diverse, naturally or artificially
756 produced mutations and their effects on the traits of interest. Such research could use
757 all of the approaches described above and higher-throughput assays that could be
758 developed with improved technology, for example as described in Box 3. This will
759 include generating mutations not found in known strains in nature to probe for those
760 that could be involved in human adaptation in the future(180, 181). The goal would be
761 to identify classes of functionally equivalent substitutions, sufficient individually or in
762 defined combinations to confer a trait of interest when introduced into a defined, avian-
763 adapted genetic background. Use of *in vitro* approaches with noninfectious viruses or
764 viral components, or infectious viruses containing surface proteins to which there was
765 already population-wide immunity would reduce the possible biosafety and biosecurity
766 risks of such studies (182).

767 The second approach is to develop and improve the throughput and accuracy of
768 biochemical and cell-biological assays of these traits, so that virus isolates can be

769 characterized phenotypically in a routine manner, reducing reliance on sequence-based
770 predictions. It seems feasible to develop high-throughput versions of many of the assays
771 described in this review for each of the three traits discussed, which could then be
772 routinely run on surveillance isolates to contribute to risk prediction. For none of these
773 three traits is there a single gold standard assay, and different assays may provide
774 different estimates of risk.

775 The third approach is to improve animal models to more precisely study phenotypes
776 that are important for human adaptation, and to clarify whether the notion of
777 "mammalian adaptation" is in fact a valid category. Ferrets are the closest known model
778 for human transmission (see Box 4). Respiratory-droplet transmission studies in ferrets,
779 and potentially in other animal models, have shown a remarkably strong correlation
780 with human transmissibility of influenza A strains (25). While these assays are not
781 perfectly predictive, they may be the most reliable way at present to assess the
782 transmission potential of a virus in human populations. Here a partial counterexample
783 to their overall strong predictive value is H7N9 avian influenza isolates from human
784 zoonotic cases. These viruses transmit in ferrets, albeit less efficiently than human
785 seasonal strains, yet human-to-human transmission has been extremely rare in the
786 hundreds of human zoonotic cases caused by H7N9 (25). A challenge is the expense and
787 practical challenge of using large enough numbers of ferrets (25, 183, 184) to assess
788 transmissibility; this will remain a technique of limited throughput for the foreseeable
789 future. Nonetheless, the value of ferret testing for risk assessment can be enhanced in
790 at least two ways: first, by standardizing the conditions for ferret transmission

791 experiments, so these can be more confidently compared between laboratories; and
792 second, by continuing to combine ferret studies with studies of viral traits and
793 sequence/structural studies to further identify correlates of transmissibility in ferrets.

794 While the biological properties of a virus certainly play a large role in determining the
795 pandemic risk posed by a strain, it is possible that even a virus perfectly adapted for
796 human-to-human transmission might fail to transmit extensively, due to ecological
797 factors, chance, or both. Initiation of a pandemic requires not only a well-adapted virus
798 but ecological opportunity to spill over into humans (perhaps multiple times if the first
799 introduction is not "successful" (185)), as well as a human population that is
800 immunologically susceptible and sufficiently connected to establish ongoing
801 transmission (Box 5). Additional complexity arises from the fact that selection pressures
802 for within-host proliferation and competition may diverge from those needed for
803 efficient transmission (61). Genetic bottlenecks at the time of transmission (47, 48, 66)
804 may further enhance the role of chance, as a highly adaptive variant arising in a host
805 may not get transmitted in a particular event. However, selective bottlenecks, which
806 have been observed in experimental transmission of H5N1 and avian-like H1N1 viruses
807 in ferrets, could lower the barrier to emergence of human-adapted viruses (64, 65).
808 Both ecological and host factors are considered in the CDC's IRAT (45).

809 Evolutionary factors also play a role in pandemic risk evaluations. Even with excellent
810 surveillance, we may never isolate exactly the virus that is destined to cause a pandemic
811 from an animal reservoir or a zoonotic human case; rather, we may isolate its

812 evolutionary precursor. Understanding the potential of a strain to produce pandemic-
813 capable progeny is yet a further scientific challenge. Perhaps the most startling finding
814 of the gain-of-transmissibility experiments with H5N1 avian influenza viruses was that
815 so few mutations were required to convert a strain circulating in birds to mammalian
816 transmission. This concern was reinforced by a finding that many of these mutations,
817 including combinations of some of them, were already present in strains isolated during
818 surveillance (32). The interpretation of the latter finding, however, is complicated by the
819 problem of epistasis: the effect of these mutations in the genetic background of field
820 strains may or may not be the same as in the strain studied in the laboratory.

821 It seems clear that a pandemic risk assessment informed by genetic sequence data is
822 better than one uninformed by such sequence data, but the thought experiment of
823 considering the 2009 swine-origin virus, had it been seen prior to initiating the
824 pandemic, shows that such efforts may fail to identify the risk posed by strains that
825 subsequently cause a pandemic. According to the knowledge at the time, early human
826 isolates of 2009 (and presumably their swine precursors) would have had an HA
827 intermediate between human and avian adaptation in terms of receptor binding. They
828 had an activation pH outside the range previously seen in human viruses and more
829 typical of avian viruses. Moreover, these viruses lacked the amino acid residues then
830 thought to confer human adaptation for the polymerase complex. We must imagine
831 that had this strain been detected in swine surveillance prior to the pandemic
832 emergence, genetic as well as phenotypic considerations would have marked it as low
833 risk, creating a false-negative risk assessment. Given that this virus did in fact create a

834 pandemic, it is evident that failure of a nonhuman influenza virus to fully meet the three
835 criteria discussed in this review does not disqualify it from posing a significant risk of a
836 pandemic.

837 Whether false positive predictions of high pandemic risk are also possible is more
838 difficult to determine, because even a strain that is truly high risk may fail to cause a
839 pandemic for any number of reasons; thus it is challenging to prove that an assessment
840 of high risk for a particular strain was erroneous. From a decision-making perspective, a
841 false positive is perhaps less worrisome than a false negative, as a false positive may
842 motivate expenditure on prevention measures directed at a strain that would not have
843 caused a pandemic, while a false negative may lead to a failure to respond to a strain
844 that would.

845 As we seek to improve our understanding of genetic and phenotypic bases of efficient
846 human transmission of influenza viruses, there are multiple possible approaches. One
847 approach that has received considerable attention recently is to perform gain-of-
848 transmissibility studies in highly pathogenic avian viruses; this has been controversial
849 because of concerns about the unusual biosafety and biosecurity risks entailed in such
850 studies (for contrasting perspectives on these risks, see the exchange in 2014-5 between
851 Lipsitch and Inglesby, and Fouchier (182, 186, 187)).
852
853 There are alternative approaches to ferret gain-of-transmission experiments in highly
854 pathogenic avian influenza viruses, though disagreement remains about the level of

855 evidence such alternatives provide. One alternative is to perform similar experiments
856 starting from avian viruses that are not highly pathogenic in mammals, and/or are
857 related to currently circulating human seasonal viruses, so that immunity would already
858 be present in the population. Such experiments can provide the same degree of causal
859 rigor as gain-of-function in highly pathogenic avian viruses with novel surface antigens
860 and can elucidate general principles of mammalian adaptation, but they cannot confirm
861 that the same changes would be observed in other strains that are not used in the
862 experiment. A recent report shows a related way forward: the recreation of the steps of
863 mammalian adaptation using viruses whose HA and NA are already circulating in
864 humans (87). Such loss+gain-of-transmissibility experiments reconstruct the properties
865 of naturally occurring seasonal human strains, from laboratory-generated, avian-
866 adapted (or at least human-deadapted) precursors. Reconstructing such seasonal strains
867 should pose a risk similar to that of working with the seasonal strains themselves, less
868 than that of a novel subtype. A 2011 report employed a similar strategy, demonstrating
869 the importance of HA activation pH in mouse adaptation by selection experiments on a
870 live attenuated H5N1 vaccine strain lacking the NS1 gene (188). More recently, a 2009
871 H1N1 pandemic virus was modified to express a mutation that increased pH of HA
872 activation, then selected in ferrets for droplet transmission, and it was found that a
873 second site mutation partially restored the lower pH of activation of the selected virus
874 (134). One limitation of de-adaptation strategies is that the acquisition of
875 transmissibility is perhaps most likely to evolve by reversion of the de-adaptation
876 changes. As with all gain-of-function and loss-of-function studies, epistatic effects of

877 other loci in the genetic background of the viruses used in such studies set limits to the
878 generalizability of such experiments. Another kind of alternative is simple
879 characterization of ferret transmissibility of naturally occurring highly pathogenic strains
880 without selection for airborne transmission. This approach can provide correlative
881 evidence for the importance of genetic differences but cannot prove the mechanistic
882 role of any particular change.

883 Even if strain-based assessment methods were much better, surveillance would be a key
884 rate-limiting step for pandemic risk assessment to direct countermeasure development.
885 If a virus about to cause a pandemic is not found in surveillance it cannot be assessed.
886 The fact that we have yet to identify a pandemic strain in nonhuman hosts or in human
887 spillover cases before the pandemic starts indicates there is much work to be done.
888 Although surveillance has expanded since the 2009 pandemic, it has not been designed
889 to optimize the chances of detecting a pandemic strain before it becomes pandemic;
890 indeed, how to do so is not clear at present. Some possible considerations would be to
891 maximize the diversity of isolates collected, to preferentially sample strains that are
892 known to cause human infections, and to feed back information from risk assessments
893 to inform choice of sampling. Rapid sequencing and phenotypic characterization of
894 strains and dissemination of this information, along with interpretations of the risk
895 profiles implied, is also important to maximizing the value of surveillance. Further
896 thought should be given to the possibility of using high-throughput sequencing as a
897 screen for which viruses should be subjected to phenotypic testing, which for the
898 moment is typically more costly, slower, and lower-throughput than sequencing. More

899 deliberate approaches to the design of surveillance systems would also depend on
900 answering the question addressed in Box 2: how different must a virus be from
901 previously characterized viruses to merit separate evaluation of its pandemic risk? The
902 uncertainties noted above about the phenotypic characteristics of viruses isolated from
903 previous pandemics (which may have been present in the primary isolate or may have
904 arisen during egg passage in the laboratory) underline the need for careful attention to
905 passage histories of surveillance isolates to avoid altering their genotype and phenotype
906 post-isolation (189). Expanding and rationalizing surveillance in this way would require
907 overcoming political, logistical and financial constraints that vary between countries and
908 regions.

909 Even with all of the foregoing suggestions in place, it may be improbable that we can
910 reliably identify the "needle in the haystack" that is the next pandemic influenza strain.
911 Ultimately, the goal is not risk assessment for its own sake, but preparedness and early
912 response to pandemic threats. In other areas where security is at stake, it has been
913 argued that making and improving predictions should be accompanied by a systematic
914 effort to design responses that will not fail even if the predictions are wrong (190). In
915 the influenza context, the value of some countermeasures is strongly reliant on our
916 ability to identify truly high-risk prepandemic threats: notably, preparation of seed
917 vaccine stocks for candidate pandemic strains, stockpiling of subtype-specific vaccines,
918 and culling of poultry infected with such strains. Other types of countermeasures,
919 ranging from strengthening local public health departments to stockpiling antivirals or
920 ventilators to developing faster processes for vaccine manufacture to universal vaccines

921 that should be effective against any influenza A strain, should provide benefits whether
922 or not we have advance notice of the strain causing the next pandemic. A
923 comprehensive assessment of priorities to prevent or mitigate the next influenza
924 pandemic should consider the balance between improving our risk assessment capacity
925 and developing responses robust to the possibility that we will once again be caught by
926 surprise.

927

928

929 ACKNOWLEDGMENTS

930 We thank Jesse Bloom, Ruben Donis, Judith Fonville, Scott Hensley, and Benjamin
931 TenOever for contributing valuably to the workshop on which this paper is based and
932 Ruben Donis for significant assistance in improving the manuscript. We thank James Hay
933 for the Figure for Box 5. This work and the workshop from which it originated were
934 supported in part by the Research and Policy for Infectious Disease Dynamics (RAPIDD)
935 program of the Science and Technology Directorate, U.S Department of Homeland
936 Security, and the Fogarty International Center, NIH. ML's work on this paper was
937 supported by MIDAS Center of Excellence Cooperative Agreement U54GM088558 from
938 the National Institute of General Medical Sciences, NIH. CAAB was supported by
939 Discovery Grant (355837-2013) from the Natural Sciences and Engineering Research
940 Council of Canada (NSERC, http://www.nserc-crsng.gc.ca) and an Early Researcher
941 Award (ER13-09-040) from the Ministry of Research and Innovation of Ontario
942 (http://www.mri.gov.on.ca). SMS was supported by a joint A*STAR-NHMRC grant with
943 number 12/1/06/24/5793. CJR was supported by the NIH, National Institute of Allergy
944 and Infectious Diseases (NIAID), Centers of Excellence for Influenza Research and
945 Surveillance (Contract HHSN272201400006C). COW was funded in part by NIH R01
946 GM088344. PK was funded by research grant NIH R01 GM098304. CAR is supported by a
947 University Research Fellowship from the Royal Society. PRM is funded by the UK Medical
948 Research Council (Grant reference number G0801822). SR was supported by Wellcome

949 Trust Project (UK, Project 093488/Z/10/Z), Wellcome Trust Collaborator Award (UK,
950 200187/Z/15/Z), Wellcome Trust Investigator Award (UK, 200861/Z/16/Z), Medical
951 Research Council Project (UK, MR/J008761/1), and National Institute for General
952 Medical Sciences (US, MIDAS U01 GM110721-01). SH is supported by a VIDI grant from
953 the Netherlands Organisation for Scientific Research (NWO; contract number
954 91715372).
955
956 The findings and conclusions in this report are those of the authors and do not
957 necessarily reflect the views of the Centers for Disease Control and Prevention, the
958 Department of Health and Human Services or its Components, or the NIH or its
959 institutes.
960
961 COI
962
963 During the prepartion of this manuscript, CAAB received funding through a
964 research contract with AstraZeneca Inc., but for distinct, unrelated
965 research. AstraZeneca Inc. had no role in the preparation of this work or
966 in the decision to submit the work for publication.
967
968 ML reports the following financial disclosures for topics unrelated to this manuscript:
969 consulting income from Pfizer and Affinivax (both donated to charity) and research

970 funding from Pfizer and PATH Vaccine Solutions. These entities had no role in the
971 preparation of this work or in the decision to submit the work for publication
972
973 All other authors report no conflicts of interest.

974

975 BOXES

976 **BOX 1 Steps in pandemic emergence**

977 For an avian-adapted strain of influenza A to become a pandemic strain, several events
978 are required:

979 1. The avian-adapted strain must become sufficiently widespread in wild or domestic
980 birds, swine or other reservoir species to expose at least one human to infection.

981 2. One or more humans must acquire infection from the reservoir species.

982 3. The infection must replicate sufficiently in a zoonotic case to produce infectious virus
983 in respiratory or other secretions.

984 4. The infection must be transmitted to additional humans, avoiding an "early"
985 termination of the transmission chain due to chance. Such early termination is a
986 significant risk given the relatively low infectiousness of influenza and the moderate
987 degree of overdispersion in the number of secondary cases infected by each case, both
988 of which contribute to the probability that a transmission chain will terminate by chance
989 (191, 192). It must also avoid extinction due to deliberate control efforts put in place by
990 public health authorities (193, 194).

991 5. Finally, the infection must spread beyond the local area to infect members of distant
992 populations, a process accelerated by modern global travel (195). This step and the one

993 before are enhanced if the level of population susceptibility is high, as occurs when the
994 surface proteins of the new strain are dissimilar to those on any currently or recently
995 circulating human influenza A strains.

996 We know from serologic studies and human infections that several different influenza A
997 viruses have achieved steps 1 and 2 at any given time (15, 16). Steps 3 and/or 4 appear
998 to be the rare, rate-limiting steps; that is, the conditional probability of achieving step 3
999 and 4 given the previous steps is low, so that sustained human-to-human transmission
1000 of a novel strain occurs a few times per century while zoonotic infections must occur
1001 thousands or more times per year. No case is known in which an influenza A strain has
1002 reached stage 4 but failed to reach stage 5, although it may have happened undetected.

1003 The appearance -- by mutation or reassortment -- and selection of genetic changes that
1004 encode human-adaptive viral traits may be seen as a process that can accelerate or
1005 increase the probability of one or more of these steps (though there is no guarantee
1006 that a given change that enhances one of these steps will enhance all of them . This is
1007 why the detection of phenotypes associated with human adaptation in avian or zoonotic
1008 isolates of novel influenza A viruses is thought to correlate with increased risk of
1009 pandemic emergence. As we describe throughout the paper, the process of human
1010 adaptation need not be complete to initiate a pandemic, so it may continue to occur at
1011 various stages throughout this progression.

1012

BOX 2 Granularity of pandemic risk prediction – for what taxonomic level does it make sense?

Determining the appropriate taxonomic level for influenza virus risk assessment is a challenging endeavor. Influenza virus subtype is a convenient classification but there can be substantial variation in estimable risk within subtype. For example, H5N1 viruses can be roughly segregated into high pathogenicity and low pathogenicity phenotypes with the high pathogenicity variants generating substantially greater concern for both human and animal populations. Even within the high pathogenicity H5N1 variants, risk to animal populations and potential for adaptation to humans is likely to vary by phylogenetic lineages or clades of viruses. Much of the difficulty for predicting the threat posed by subtypes or coarse grained concepts of virus variants stems from two factors: first, a lack of understanding of how genetic context affects the ability of a virus to adapt for efficient spread in humans; and second, the critical, and geographically variable, role of ecology in determining likelihood of cross species transmission.

Phylogenetic clade is a practical unit for risk prediction. However, in species where reassortment is frequent, phylogenetic clade must be considered on a gene by gene basis. The definition of phylogenetic clades can be challenging and arbitrary, but recent efforts to develop a unified nomenclature for highly

pathogenic H5N1 viruses may offer a transferrable framework for the classification of other viruses(196, 197). Clades of viruses circulating in poultry, swine or other domestic animals with extensive human interactions should be prioritized for surveillance and further study. Foundational efforts are required to assess the diversity of viruses present in these animal populations, particularly for low pathogenic avian influenza viruses. Further study will then be required to assess the abundance and prevalence of different virus subtypes and clades, along with geographic spread and overlap with ecological risk factors(198, 199), e.g. live animal markets, cohabitation of humans and animals, and biosecurity in animal processing facilities.

Antigenic characterization of animal influenza viruses should form part of a comprehensive risk assessment, particularly of viruses from swine and possibly dogs. Swine influenza virus diversity is driven in large part by introductions of viruses from humans to swine (200-202). The substantial antigenic diversity of viruses circulating in swine and antigenic differences with viruses circulating in humans poses an ever increasing risk for re-introduction into humans. Much of the antigenic variation in swine has a strong relationship to phylogenetic clade (202). Similarly, the high contact rates between humans and dogs, combined with increased circulation of H3N2 canine influenza viruses, may present increasing opportunities for reassortment (203) and for zoonotic infections (204).

BOX 3: Novel approaches to identifying genomic predictors of traits and transmission phenotypes

The advent of inexpensive, large-scale sequencing, combined with improved computing power and novel algorithms to interpret nucleotide and protein sequences, have generated new approaches to characterizing the genotype-trait and genotype-transmission phenotype maps in influenza viruses. Some are well-established, while others are under active development. They include:

Protein structural analysis to identify properties of individual amino acid residues and pairs of residues. A number of approaches have been devised to make use of databases of genome sequences and inferred protein sequences of influenza virus isolates, alone or in combination with metadata on the source (species), date of isolation and passage history of the isolates.

Characterizing the predictors – at the level of individual amino acid residues within a protein – of variability or conservation can assist in identifying the major selection pressures on that protein. Evolutionary analysis of the predictors of high rates of nonsynonymous substitutions within HA showed solvent accessibility and proximity to the sialic acid receptor binding site are the strongest predictors of high nonsynonymous evolutionary rates (205). Comparisons of residue-specific evolutionary rates in avian and human lineages can help to assess which sites are specifically involved in human adaptation and which may be evolving in avian reservoirs with potential consequences for human adaptation (206).

Innovative use of metadata associated with sequences deposited in databases will be required to ensure that computational inferences from these databases are reliable. For example, methods that aim to identify sites under positive selection in the HA protein frequently find regions or sites that seem to contradict experimental evidence (205, 207, 208). Several of these apparent contradictions can be resolved by accounting for viral passaging. For example, passaging in regular MDCK cells produces a strong signal of positive adaptation underneath the sialic-acid binding site; this signal is entirely absent in unpassaged virus or virus passaged in SIAT1 MDCK cells (209) .At the same time, passage bias mutations are assumed to increase fitness of the strain in the respective species and are often necessary to grow in culture at all. Therefore, sites associated with isolates passaged in mammalian cultures vs. those passaged in embryonated hen's eggs have the potential to further identify sites associated with mammalian or human adaptation.

Metadata can also help to point to individual amino acids associated with human adaptation. For example, one proposed computational approach is to find potentially zoonotic human-isolated sequences when the majority of their database hits from preceding years were of animal origin. This serves, on one hand, as systematic survey to derive lists of times and places of likely zoonotic events and, on the other hand, provides close sequence pairs of zoonotic human and their putative animal precursors. In those pairs, common sites that repeatedly changed from the animal to the human zoonotic isolates could be reasoned as being involved in human adaptation. Combining

these sites with those from passage changes, provides strong evidence for the involvement of a particular site in host adaptation.

Network analysis of the level of sequence covariation of pairs of residues among protein sequences in the database has led to the identification of groups of mutually covarying sites, which have been used to define features of the HA protein that play a role in determining glycan receptor usage (210, 211). Complementary to such covariation analysis is the analysis of predicted molecular interactions. Using X-ray co-crystal structures or modeled structural complexes of HA-glycan receptors, molecular features have been defined as distinct networks of inter-residue interactions involving key residues that make contacts with the different glycan receptor topologies. These features go beyond hallmark residue analyses and more accurately predict how amino acid variations in the receptor binding site impact the inter-residue interactions and glycan receptor binding specificity (56). Similarly, network analysis of amino acid residues predicted to have significant interactions has shown that antigenic sites on the HA interact with residues controlling glycan receptor binding specificity, and that changes in these antigenic residues can then lead to changes in receptor-binding affinity (212).

It seems likely that as these different lines of evidence – structural location, biophysical interaction, sequence covariation, sequence evolutionary rates, association with zoonotic or in vitro adaptation, etc.—begin to be understood at the resolution of individual amino acids within an influenza protein, such overlapping approaches will

1120 yield clearer understanding of the genetic and structural bases of host adaptation to
1121 human infection and transmission. A significant step toward such integration is the
1122 recent release of the FluSurver online tool which automatizes influenza sequence and
1123 structure analysis and highlights mutations that could alter the discussed traits based on
1124 extensive literature-derived genotype to phenotype lists, structural visualization of the
1125 mutation positions and their geographic and temporal frequency of occurrence and co-
1126 occurrence for epidemiological relevance (http://flusurver.bii.a-star.edu.sg and directly
1127 from within GISAID http://www.gisaid.org). In particular, the tool has been successful in
1128 picking up mutations affecting host receptor binding (213) as well as pH dependency
1129 (132, 133). However, also in this approach, annotations of the effects of mutations are
1130 based on inference from similarity to mutations studied in specific sequence contexts,
1131 which in most cases will not be identical to the investigated input sequences.

1132 *Association studies.* Understanding the genetic basis of adaptive phenotypic change is a
1133 central goal in biology, and influenza poses special challenges and advantages relative to
1134 other organisms. Association studies have begun mapping the genomes of *Arabidopsis*
1135 *thaliana* to over 107 quantitative traits and the genomes of humans to over 100,000
1136 (214, 215). These studies often investigate genetic variation at the scales of single
1137 nucleotide polymorphisms, alleles, and loci. Motif-based approaches have already
1138 proven useful in influenza (e.g., the insertion of multiple basic amino acids indicates
1139 highly pathogenic H5 and H7), and such simple, robust correlations simplify the
1140 prediction of phenotypic traits. Recent investigations of influenza (181, 216, 217) have
1141 shown that many mutations have roughly consistent impacts across diverse

backgrounds. A complication of all association studies is confounding from genetic linkage and diverse environmental selective pressures. Although influenza's genes might be tightly linked over short time scales, the virus evolves quickly, and many traits can be assumed to be under stabilizing selection. Thus, association studies that appear statistically impractical now may be feasible with a few more years of expanded surveillance.

As reviewed here, however, influenza often breaks simple genetic rules, perhaps due to epistasis (e.g., (54)). High-dimensional genotype-phenotype relationships obscure simple correlations from association studies. A relevant lesson comes from The Cancer Genome Atlas (TCGA), which amassed sequences from thousands of diverse tumors to investigate the mutations leading to different cancer types. Although metastatic cancers are typically conceptualized as possessing six main phenotypic traits (218), TCGA revealed that the genetic commonality among tumors of any given type is shockingly low (219, 220). Human genomes are much larger and more complex than influenza's, however, and so it is possible that an influenza atlas might reveal more patterns, which could inspire hypothesis-driven experiments (221).

High-throughput, large-scale screens of mutational effects on hemagglutinin receptor binding. Binding of upper-respiratory-tract glycans by the influenza virus hemagglutinin is one of the best-understood ingredients in making a virus capable of efficient human transmission. Yet the viral sequence determinants of this trait have been mapped only for a limited number of variants. A systematic screening strategy to scan the genetic

"landscape" for sequences with a preference for human glycan receptors might include four components: 1) selection of viral genetic background, 2) large-scale mutagenesis, 3) screening and selection, and 4) confirmatory assays. Because both mutations near and far from the sialic-acid-binding site on hemagglutinin have been shown to alter glycan specificity, this should be based on a minimally biased approach to mutagenesis: screening combinations of all possible substitutions at all hemagglutinin residues that are not absolutely conserved across known subtypes. Critical considerations include choice of viral genetic background (both subtype and strain identity), extent of mutagenesis (if conserved sites are omitted, up to 4 simultaneous mutants can be screened with some effort), and design of highly parallel screening, selection, and confirmatory assays. The mutagenesis and screening involved would be extremely large in scope: (before eliminating conserved residues, ~550 residues x 20 amino acids)^4 = 1.4 x 10^{16} variants for each subtype tested and would thus require substantial effort. However, some computational pre-screening to narrow the set of residues tested combined with contemporary mutagenesis and screening technologies such as deep scanning codon mutagenesis (181, 222, 223) make such an endeavor feasible.

BOX 4: Ferret model: validity and limitations in pandemic risk assessment

The use of small mammalian models in influenza virus pathogenesis and transmission has proven invaluable for the study of these complex, polygenic traits. The ferret model is particularly valuable, as ferrets are highly susceptible to most influenza A viruses without the need for prior host adaptation. However, even this gold-standard model is not a true substitute for humans. Below, we summarize the benefits, drawbacks, and alternatives to the ferret model for the study of influenza.

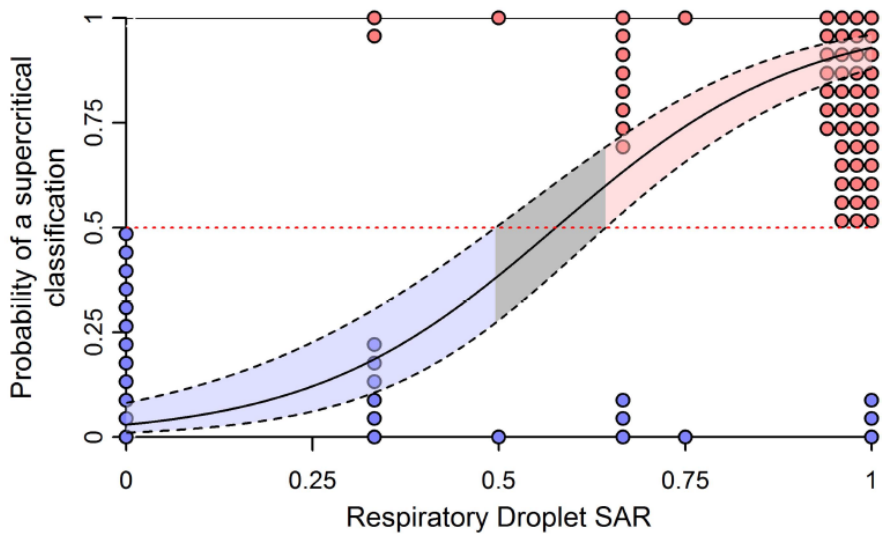
Validity. Influenza is a respiratory pathogen in humans, and employing mammalian models that possess comparable lung physiology permits a greater extrapolation of results from the laboratory. Importantly, the linkage types and distribution of sialic acids throughout the ferret respiratory tract are generally comparable to humans (224, 225): like humans, ferrets express the sialic acid N-acetylneuraminic acid (Neu5Ac), but not the sialic acid N-glycolylneuraminic acid (Neu5Gc), on respiratory epithelia. As a result, ferrets are uniquely suited for the study of influenza viruses compared with other small mammalian models which express Neu5Gc (226). Furthermore, human and avian influenza viruses exhibit comparable binding to upper and lower respiratory tract tissues in ferrets and humans (84, 85).

Secondly, ferrets infected with influenza viruses demonstrate numerous clinical signs and symptoms of infection associated with human disease. Ferrets infected with human

1204 influenza viruses often exhibit transient weight loss, transient fever, and sneezing,
1205 whereas infection with selected HPAI viruses in this species can lead to pronounced
1206 weight loss, sustained fever, lethargy, dyspnea, and neurological complications (227).
1207 Thus, ferrets represent a preclinical model to assess the ability of novel vaccine and
1208 antiviral treatments to mitigate influenza virus. As ferrets are a suitable model for the
1209 coincident study of pathogenesis and transmission, this model allows for a greater
1210 understanding of virus-host interactions and the interplay between both of these
1211 parameters.
1212
1213 Finally, the ferret model can yield valuable insights about the potential human-to-
1214 human transmissibility of influenza viruses – the critical determinant of pandemic risk. A
1215 recent meta-analysis showed that estimates of transmissibility derived from ferret
1216 respiratory droplet transmission studies could explain 66% of measured variation in
1217 human transmissibility, for influenza subtypes that have been detected in humans (25).
1218 Furthermore, there is a strong statistical relationship between the attack rates
1219 measured in particular ferret experiments and the probability that the influenza strain in
1220 question is capable of sustained transmission among humans: if two-thirds or more of
1221 contact ferrets become infected via respiratory droplets, then the strain is likely to have
1222 pandemic potential (see figure). However, extrapolation of this relationship to novel
1223 strains is inherently risky, and variable outcomes observed for H7N9 influenza
1224 transmission in ferrets highlight the potential for false alarms. Further analysis of ferret

1225 transmission experiments, ideally in concert with molecular and virological research,
1226 could raise their sensitivity and specificity for identifying pandemic threats.
1227
1228 *Limitations.* There is no 'perfect' small mammalian model for influenza. A longstanding
1229 challenge of the ferret model has been limited availability of ferret-specific commercial
1230 reagents compared with other models, though recent sequencing of the ferret genome
1231 should improve this situation (228). Ethical considerations, and the size and cost of
1232 ferrets, necessitate generally small sample sizes in ferret experiments, limiting statistical
1233 power (183). Like other vertebrate models, the ferret is not appropriate for high-
1234 throughput screens, so research in the ferret model is most potent when complemented
1235 with *in vitro* and computational approaches. Finally, ferrets are not well suited to model
1236 the multiple influenza exposures over several years that may be experienced by humans
1237 and may mold their immune responses in ways that affect the infection risk with
1238 subsequent viruses (229). Studies of first influenza infection in ferrets may thus
1239 overestimate infection and/or transmission risk relative to that in populations with a
1240 history of prior infection with related viruses (230).
1241
1242 *Alternatives.* The ferret is but one of several well-characterized mammalian models for
1243 influenza virus. Mice are widely used in the field as they offer a greater availability of
1244 commercially available species-specific reagents, permit studies with greater statistical
1245 power due to larger sample sizes, and offer the advantage of transgenic animals.
1246 However, not all human influenza viruses replicate well in mice without prior adaptation

1247 due to a predominance of avian-like receptors in the murine respiratory tract; also mice
1248 do not display clinical signs and symptoms of influenza that mimic humans, and are not
1249 a reliable model for virus transmission studies. The guinea pig is another model, and
1250 offers several comparable advantages to ferrets, including generally similar lung
1251 physiology to humans and potential for transmission studies. Experiments in guinea pigs
1252 are often less expensive than in ferrets, because of lower husbandry costs and reduced
1253 drug costs when dosing is based on body weight (231). However, guinea pigs do not
1254 exhibit clinical signs and symptoms of infection similar to humans, and do not exhibit
1255 severe disease following infection with HPAI or pandemic influenza viruses, limiting their
1256 utility for viral pathogenesis studies.
1257



1258

1259 **Figure for Box 3:** Ferret respiratory droplet transmission experiments predict the
1260 potential for sustained human-to-human transmission of influenza viruses. The solid
1261 line shows the weighted logistic regression relationship predicting the probability that a
1262 given strain is supercritical (i.e. capable of sustained spread among humans), and the

dashed lines show the 95% confidence interval for the prediction. Filled circles show the measured secondary attack rates (SAR) in ferrets for influenza subtypes that are known to be subcritical (blue) or supercritical (red) in humans. The filled pink area shows the range of SAR for which the virus is significantly likely to be supercritical. Reprinted from (25).

BOX 5: Role of seroepidemiology in pandemic risk assessment

Pandemic threat assessment can also be enhanced by immunological surveys of human populations in geographical area where strains of concern are known to be circulating (232). Serological surveys can help to estimate the frequency of spillover infections from non-human to human hosts and also to assess the degree of cross reactivity arising from endemic human strains that share recent genomic ancestors with non-human strains of concern (Figure for Box 4).

Attempts have been made to use serological surveys to estimate the rate of spillover infections to humans for recent strains of concern (233, 234). Sometimes blood samples are obtained from the general population (235) and other times only high risk individuals are tested. Inherent measurement error and cross-reactivity between human and non-human strains make the measurement of low rates of incidence problematic (234). Confidence that serologic responses truly reflect zoonotic transmission, rather than cross-reactivity with antibodies generated in response to human influenza infection, may be enhanced by comparison of high-risk persons to those without known exposure to zoonotic sources (236, 237). Although there is evidence of exposure of poultry workers to H5N1 influenza viruses in China, rates are much lower than for other endemic non-human influenza viruses(238), such as H9N2 (239). More recent studies of exposure of high risk workers to the H7N9 lineage suggest even higher rates of exposure to this new strain than has been observed in similar studies of H5N1 or H9N2(240).

Even when rates of spillover can be estimated accurately, the use of such information in pandemic threat assessment is not obvious. Clearly, the first detected presence of human infections for a given strain is of concern because the degree of transmissibility among humans is unknown. Should the emergent strain fail to achieve sustained transmission, it is not immediately clear how best to use further information on the frequency of human spillover infections. For example, should we interpret high sustained levels of human spillover as evidence of increased risk because of the number of human infections, or as evidence of decreasing risk because of the number of times the strain has failed to achieve sustained transmission?

Cross reactivity between non-human and human influenza strains has implications beyond the measurement of spillover infections. Often levels of cross reactivity in humans may indicate some degree of reduced population susceptibility (23). All else equal, such evidence of lower population susceptibility should reduce our level of concern about a pandemic threat from a particular virus, because even if it gains efficient human-to-human transmissibility, its effective reproductive number and the proportion of the population at risk will be less than for a virus to which there is no cross-reaction in the population. For example, older individuals are thought to have been far less susceptible to pandemic H1N1 than were younger individuals, because they had previously been exposed to similar strains early in life (241). The low average age of infection with a swine variant form of H3N2 (H3N2v) in North America (8) is likely driven by reduced susceptibility in adults because of early exposure to similar strains. Such immunological overlaps are likely to be a general feature of influenza emergence

because human strains frequently emerge into swine populations (200).

Data on reduced human susceptibility due to cross-reactivity must be synthesized with other data used for threat assessment. In some cases, the aging of the part of the population with prior exposure to a closely related strain could be the most important known factor increasing the risk of an emergence event. Mechanistic models could be used to estimate the degree of increased risk of emergence due to the aging of partially immune cohorts.

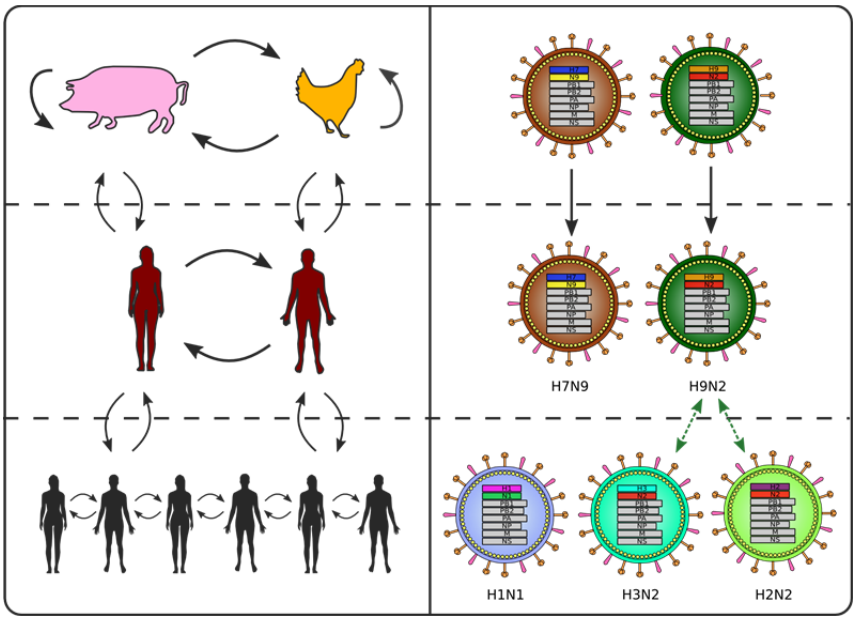


Figure for Box 5. Transmission genomics of non-human transmission (top), spillover transmission (middle) and sustained human transmission (bottom). Haemagglutinin and neuraminidase gene segments have been color-coded to show an example shared infection history in humans who are current spillover hosts for H7N9 and H9N2. These shared evolutionary histories make it challenging to interpret serological studies of human spillover infections. Humans infected by H2N2 or H3N2 will likely have cross-reactive antibodies to H9N2, because of the similarity between the neuraminidase in those viruses. Because incidence of spill-over infection is likely to be low, even low-levels of cross-reactivity can make the interpretation of serological studies of the general population challenging.

1. **Krauss S**, **Webster RG**. 2010. Avian influenza virus surveillance and wild birds: past and present. Avian Dis **54**:394–398.

2. **Vandegrift KJ**, **Sokolow SH**, **Daszak P**, **Kilpatrick AM**. 2010. Ecology of avian influenza viruses in a changing world. Ann N Y Acad Sci **1195**:113–128.

3. **Webster RG**, **Bean WJ**, **Gorman OT**, **Chambers TM**, **Kawaoka Y**. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56**:152–179.

4. **Hinshaw VS**, **Webster RG**, **Turner B**. 1979. Water-Borne Transmission of Influenza A Viruses? Intervirology **11**:66–68.

5. **Hénaux V**, **Samuel MD**. 2015. AVIAN INFLUENZA SHEDDING PATTERNS IN WATERFOWL: IMPLICATIONS FOR SURVEILLANCE, ENVIRONMENTAL TRANSMISSION, AND DISEASE SPREADdx.doi.org. Wildlife Disease Association.

6. **Malik Peiris JS**. 2009. Avian influenza viruses in humans. Rev - Off Int Epizoot **28**:161–173.

7. **Qin Y**, **Horby PW**, **Tsang TK**, **Chen E**, **Gao L**, **Ou J**, **Nguyen TH**, **Duong TN**, **Gasimov V**, **Feng L**, **Wu P**, **Jiang H**, **Ren X**, **Peng Z**, **Li S**, **Li M**, **Zheng J**, **Liu S**, **Hu S**, **Hong R**, **Farrar JJ**, **Leung GM**, **Gao GF**, **Cowling BJ**, **Yu H**. 2015. Differences in the Epidemiology of Human Cases of Avian Influenza A(H7N9) and A(H5N1) Viruses Infection. Clin Infect Dis **61**:563–571.

8. **Jhung MA**, **Epperson S**, **Biggerstaff M**, **Allen D**, **Balish A**, **Barnes N**, **Beaudoin A**, **Berman L**, **Bidol S**, **Blanton L**, **Blythe D**, **Brammer L**, **D'Mello T**, **Danila R**, **Davis W**, **de Fijter S**, **Diorio M**, **Durand LO**, **Emery S**, **Fowler B**, **Garten R**, **Grant Y**, **Greenbaum A**, **Gubareva L**, **Havers F**, **Haupt T**, **House J**, **Ibrahim S**, **Jiang V**, **Jain S**, **Jernigan D**, **Kazmierczak J**, **Klimov A**, **Lindstrom S**, **Longenberger A**, **Lucas P**, **Lynfield R**, **McMorrow M**, **Moll M**, **Morin C**, **Ostroff S**, **Page SL**, **Park SY**, **Peters S**, **Quinn C**, **Reed C**, **Richards S**, **Scheftel J**, **Simwale O**, **Shu B**, **Soyemi K**, **Stauffer J**, **Steffens C**, **Su S**, **Torso L**, **Uyeki TM**, **Vetter S**, **Villanueva J**, **Wong KK**, **Shaw M**, **Bresee JS**, **Cox N**, **Finelli L**. 2013. Outbreak of variant influenza A(H3N2) virus in the United States. Clin Infect Dis **57**:1703–1712.

9. **Fouchier RAM**, **Schneeberger PM**, **Rozendaal FW**, **Broekman JM**, **Kemink SAG**, **Munster V**, **Kuiken T**, **Rimmelzwaan GF**, **Schutten M**, **Van Doornum GJJ**, **Koch G**, **Bosman A**, **Koopmans M**, **Osterhaus ADME**. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA **101**:1356–1361.

10. **Wohlbold TJ**, **Hirsh A**, **Krammer F**. 2015. An H10N8 influenza virus vaccine strain

and mouse challenge model based on the human isolate A/Jiangxi-Donghu/346/13. Vaccine **33**:1102–1106.

11. **Wei S-H**, **Yang J-R**, **Wu H-S**, **Chang M-C**, **Lin J-S**, **Lin C-Y**, **Liu Y-L**, **Lo Y-C**, **Yang C-H**, **Chuang J-H**, **Lin M-C**, **Chung W-C**, **Liao C-H**, **Lee M-S**, **Huang W-T**, **Chen P-J**, **Liu M-T**, **Chang F-Y**. 2013. Human infection with avian influenza A H6N1 virus: an epidemiological analysis. Lancet Respir Med **1**:771–778.

12. **Butt KM**, **Smith GJD**, **Chen H**, **Zhang LJ**, **Leung YHC**, **Xu KM**, **Lim W**, **Webster RG**, **Yuen KY**, **Peiris JSM**, **Guan Y**. 2005. Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol **43**:5760–5767.

13. **Yang Z-F**, **Mok CKP**, **Peiris JSM**, **Zhong N-S**. 2015. Human Infection with a Novel Avian Influenza A(H5N6) Virus. N Engl J Med **373**:487–489.

14. **Short KR**, **Richard M**, **Verhagen JH**, **van Riel D**, **Schrauwen EJA**, **van den Brand JMA**, **Mänz B**, **Bodewes R**, **Herfst S**. 2015. One health, multiple challenges: The inter-species transmission of influenza A virus. One Health **1**:1–13.

15. **Gomaa MR**, **Kayed AS**, **Elabd MA**, **Zeid DA**, **Zaki SA**, **Rifay El AS**, **Sherif LS**, **McKenzie PP**, **Webster RG**, **Webby RJ**, **Ali MA**, **Kayali G**. 2014. Avian Influenza A(H5N1) and A(H9N2) Seroprevalence and Risk Factors for Infection Among Egyptians: A Prospective, Controlled Seroepidemiological Study. J Infect Dis.

16. **To KKW**, **Hung IFN**, **Lui Y-M**, **Mok FKY**, **Chan ASF**, **Li PTW**, **Wong T-L**, **Ho DTY**, **Chan JFW**, **Chan K-H**, **Yuen KY**. 2016. Ongoing transmission of avian influenza A viruses in Hong Kong despite very comprehensive poultry control measures: A prospective seroepidemiology study. J Infect **72**:207–213.

17. **de Jong MD**, **Simmons CP**, **Thanh TT**, **Hien VM**, **Smith GJD**, **Chau TNB**, **Hoang DM**, **Chau NVV**, **Khanh TH**, **Dong VC**, **Qui PT**, **Cam BV**, **Ha DQ**, **Guan Y**, **Peiris JSM**, **Chinh NT**, **Hien TT**, **Farrar J**. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nature Medicine **12**:1203–1207.

18. **Gao R**, **Cao B**, **Hu Y**, **Feng Z**, **Wang D**, **Hu W**, **Chen J**, **Jie Z**, **Qiu H**, **Xu K**, **Xu X**, **Lu H**, **Zhu W**, **Gao Z**, **Xiang N**, **Shen Y**, **He Z**, **Gu Y**, **Zhang Z**, **Yang Y**, **Zhao X**, **Zhou L**, **Li X**, **Zou S**, **Zhang Y**, **Li X**, **Yang L**, **Guo J**, **Dong J**, **Li Q**, **Dong L**, **Zhu Y**, **Bai T**, **Wang S**, **Hao P**, **Yang W**, **Zhang Y**, **Han J**, **Yu H**, **Li D**, **Gao GF**, **Wu G**, **Wang Y**, **Yuan Z**, **Shu Y**. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med **368**:1888–1897.

19. **Kucharski A**, **Mills H**, **Pinsent A**, **Fraser C**, **Van Kerkhove M**, **Donnelly CA**, **Riley S**. 2014. Distinguishing Between Reservoir Exposure and Human-to-Human Transmission for Emerging Pathogens Using Case Onset Data. PLoS Curr **6**.

20. **Patterson KD**. 1986. Pandemic Influenza, 1700-1900. Rowman & Littlefield Pub Incorporated.

21. **Tong S**, **Zhu X**, **Li Y**, **Shi M**, **Zhang J**, **Bourgeois M**, **Yang H**, **Chen X**, **Recuenco S**, **Gomez J**, **Chen L-M**, **Johnson A**, **Tao Y**, **Dreyfus C**, **Yu W**, **McBride R**, **Carney PJ**, **Gilbert AT**, **Chang J**, **Guo Z**, **Davis CT**, **Paulson JC**, **Stevens J**, **Rupprecht CE**, **Holmes EC**, **Wilson IA**, **Donis RO**. 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog **9**:e1003657.

22. **Kuiken T**, **Holmes EC**, **McCauley J**, **Rimmelzwaan GF**, **Williams CS**, **Grenfell BT**. 2006. Host species barriers to influenza virus infections. Science **312**:394–397.

23. **Worobey M**, **Han G-Z**, **Rambaut A**. 2014. Genesis and pathogenesis of the 1918 pandemic H1N1 influenza A virus. Proceedings of the National Academy of Sciences **111**:8107–8112.

24. **Russell CA**, **Kasson PM**, **Donis RO**, **Riley S**, **Dunbar J**, **Rambaut A**, **Asher J**, **Burke S**, **Davis CT**, **Garten RJ**, **Gnanakaran S**, **Hay SI**, **Herfst S**, **Lewis NS**, **Lloyd-Smith JO**, **Macken CA**, **Maurer-Stroh S**, **Neuhaus E**, **Parrish CR**, **Pepin KM**, **Shepard SS**, **Smith DL**, **Suarez DL**, **Trock SC**, **Widdowson M-A**, **George DB**, **Lipsitch M**, **Bloom JD**. 2014. Improving pandemic influenza risk assessment. Elife **3**:e03883.

25. **Buhnerkempe MG**, **Gostic K**, **Park M**, **Ahsan P**, **Belser JA**, **Lloyd-Smith JO**. 2015. Mapping influenza transmission in the ferret model to transmission in humans. Elife **4**:e07969.

26. **Imai M**, **Watanabe T**, **Hatta M**, **Das SC**, **Ozawa M**, **Shinya K**, **Zhong G**, **Hanson A**, **Katsura H**, **Watanabe S**, **Li C**, **Kawakami E**, **Yamada S**, **Kiso M**, **Suzuki Y**, **Maher EA**, **Neumann G**, **Kawaoka Y**. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature **486**:420–428.

27. **Herfst S**, **Schrauwen EJA**, **Linster M**, **Chutinimitkul S**, **de Wit E**, **Munster VJ**, **Sorrell EM**, **Bestebroer TM**, **Burke DF**, **Smith DJ**, **Rimmelzwaan GF**, **Osterhaus ADME**, **Fouchier RAM**. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science **336**:1534–1541.

28. **Palese P**, **Wang TT**. 2012. H5N1 influenza viruses: facts, not fear. Proceedings of the National Academy of Sciences **109**:2211–2213.

29. **Linster M**, **van Boheemen S**, **de Graaf M**, **Schrauwen EJA**, **Lexmond P**, **Mänz B**, **Bestebroer TM**, **Baumann J**, **van Riel D**, **Rimmelzwaan GF**, **Osterhaus ADME**, **Matrosovich M**, **Fouchier RAM**, **Herfst S**. 2014. Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. Cell **157**:329–339.

30. **Davis CT**, **Chen LM**, **Pappas C**, **Stevens J**, **Tumpey TM**, **Gubareva LV**, **Katz JM**, **Villanueva JM**, **Donis RO**, **Cox NJ**. 2014. Use of Highly Pathogenic Avian Influenza A(H5N1) Gain-Of-Function Studies for Molecular-Based Surveillance and Pandemic Preparedness. MBio **5**:e02431–14–e02431–14.

31. **Schultz-Cherry S**, **Webby RJ**, **Webster RG**, **Kelso A**, **Barr IG**, **McCauley JW**, **Daniels RS**, **Wang D**, **Shu Y**, **Nobusawa E**, **Itamura S**, **Tashiro M**, **Harada Y**, **Watanabe S**, **Odagiri T**, **Ye Z**, **Grohmann G**, **Harvey R**, **Engelhardt O**, **Smith D**, **Hamilton K**, **Claes F**, **Dauphin G**. 2014. Influenza Gain-of-Function Experiments: Their Role in Vaccine Virus Recommendation and Pandemic Preparedness. MBio **5**:e02430–14–e02430–14.

32. **Russell CA**, **Fonville JM**, **Brown AEX**, **Burke DF**, **Smith DL**, **James SL**, **Herfst S**, **van Boheemen S**, **Linster M**, **Schrauwen EJ**, **Katzelnick L**, **Mosterín A**, **Kuiken T**, **Maher E**, **Neumann G**, **Osterhaus ADME**, **Kawaoka Y**, **Fouchier RAM**, **Smith DJ**. 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science **336**:1541–1547.

33. **Bi Y**, **Mei K**, **Shi W**, **Liu D**, **Yu X**, **Gao Z**, **Zhao L**, **Gao GF**, **Chen J**, **Chen Q**. 2015. Two novel reassortants of avian influenza A (H5N6) virus in China. J Gen Virol **96**:975–981.

34. **Jonges M**, **Welkers MRA**, **Jeeninga RE**, **Meijer A**, **Schneeberger P**, **Fouchier RAM**, **de Jong MD**, **Koopmans M**. 2014. Emergence of the virulence-associated PB2 E627K substitution in a fatal human case of highly pathogenic avian influenza virus A(H7N7) infection as determined by Illumina ultra-deep sequencing. J Virol **88**:1694–1702.

35. **Chen H**, **Yuan H**, **Gao R**, **Zhang J**, **Wang D**, **Xiong Y**, **Fan G**, **Yang F**, **Li X**, **Zhou J**, **Zou S**, **Yang L**, **Chen T**, **Dong L**, **Bo H**, **Zhao X**, **Zhang Y**, **Lan Y**, **Bai T**, **Dong J**, **Li Q**, **Wang S**, **Zhang Y**, **Li H**, **Gong T**, **Shi Y**, **Ni X**, **Li J**, **Zhou J**, **Fan J**, **Wu J**, **Zhou X**, **Hu M**, **Wan J**, **Yang W**, **Li D**, **Wu G**, **Feng Z**, **Gao GF**, **Wang Y**, **Jin Q**, **Liu M**, **Shu Y**. 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. Lancet **383**:714–721.

36. **Fonville JM**, **Burke DF**, **Lewis NS**, **Katzelnick LC**, **Russell CA**. 2013. Quantifying the fitness advantage of polymerase substitutions in Influenza A/H7N9 viruses during adaptation to humans. PLoS ONE **8**:e76047.

37. **Chen G-W**, **Chang S-C**, **Mok C-K**, **Lo Y-L**, **Kung Y-N**, **Huang J-H**, **Shih Y-H**, **Wang J-Y**, **Chiang C**, **Chen C-J**, **Shih S-R**. 2006. Genomic signatures of human versus avian influenza A viruses. Emerging Infect Dis **12**:1353–1360.

38. **Sha J**, **Chen X**, **Ren Y**, **Chen H**, **Wu Z**, **Ying D**, **Zhang Z**, **Liu S**. 2016. Differences in the epidemiology and virology of mild, severe and fatal human infections with

avian influenza A (H7N9) virus. Archives of Virology 1–21.

39. **Rogers GN**, **D'Souza BL**. 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology **173**:317–322.

40. **Connor RJ**, **Kawaoka Y**, **Webster RG**, **Paulson JC**. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology **205**:17–23.

41. **Stevens J**, **Blixt O**, **Glaser L**, **Taubenberger JK**, **Palese P**, **Paulson JC**, **Wilson IA**. 2006. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. Journal of Molecular Biology **355**:1143–1155.

42. **Glaser L**, **Stevens J**, **Zamarin D**, **Wilson IA**, **García-Sastre A**, **Tumpey TM**, **Basler CF**, **Taubenberger JK**, **Palese P**. 2005. A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol **79**:11533–11536.

43. **Pappas C**, **Viswanathan K**, **Chandrasekaran A**, **Raman R**, **Katz JM**, **Sasisekharan R**, **Tumpey TM**. 2010. Receptor specificity and transmission of H2N2 subtype viruses isolated from the pandemic of 1957. PLoS ONE **5**:e11158.

44. **Kaplan BS**, **Russier M**, **Jeevan T**, **Marathe B**, **Govorkova EA**, **Russell CJ**, **Kim-Torchetti M**, **Choi YK**, **Brown I**, **Saito T**, **Stallknecht DE**, **Krauss S**, **Webby RJ**. 2016. Novel Highly Pathogenic Avian A(H5N2) and A(H5N8) Influenza Viruses of Clade 2.3.4.4 from North America Have Limited Capacity for Replication and Transmission in Mammals. mSphere **1**:e00003–16.

45. **Trock SC**, **Burke SA**, **Cox NJ**. 2015. Development of Framework for Assessing Influenza Virus Pandemic Risk. Emerging Infect Dis **21**:1372–1378.

46. **Butler D**. 2012. Flu surveillance lacking. Nature.

47. **Poon LLM**, **Song T**, **Rosenfeld R**, **Lin X**, **Rogers MB**, **Zhou B**, **Sebra R**, **Halpin RA**, **Guan Y**, **Twaddle A**, **DePasse JV**, **Stockwell TB**, **Wentworth DE**, **Holmes EC**, **Greenbaum B**, **Peiris JSM**, **Cowling BJ**, **Ghedin E**. 2016. Quantifying influenza virus diversity and transmission in humans. Nat Genet **48**:195–200.

48. **Varble A**, **Albrecht RA**, **Backes S**, **Crumiller M**, **Bouvier NM**, **Sachs D**, **García-Sastre A**, **tenOever BR**. 2014. Influenza A virus transmission bottlenecks are defined by infection route and recipient host. Cell Host Microbe **16**:691–700.

49. **Hoye BJ**, **Munster VJ**, **Nishiura H**, **Klaassen M**, **Fouchier RAM**. 2010. Surveillance of wild birds for avian influenza virus. Emerging Infect Dis **16**:1827–1834.

50. **Sanicas M**, **Forleo E**, **Pozzi G**, **Diop D**. 2014. A review of the surveillance systems of influenza in selected countries in the tropical region. Pan Afr Med J **19**:121.

51. **Simonsen L**, **Spreeuwenberg P**, **Lustig R**, **Taylor RJ**, **Fleming DM**, **Kroneman M**, **Van Kerkhove MD**, **Mounts AW**, **Paget WJ**, **GLaMOR Collaborating Teams**. 2013. Global mortality estimates for the 2009 Influenza Pandemic from the GLaMOR project: a modeling study. PLoS Med **10**:e1001558.

52. **Kryazhimskiy S**, **Dushoff J**, **Bazykin GA**, **Plotkin JB**. 2011. Prevalence of epistasis in the evolution of influenza A surface proteins. PLoS Genet **7**:e1001301.

53. **Gong LI**, **Bloom JD**. 2014. Epistatically interacting substitutions are enriched during adaptive protein evolution. PLoS Genet **10**:e1004328.

54. **Bloom JD**, **Gong LI**, **Baltimore D**. 2010. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science **328**:1272–1275.

55. **Wu NC**, **Du Y**, **Le S**, **Young AP**, **Zhang T-H**, **Wang Y**, **Zhou J**, **Yoshizawa JM**, **Dong L**, **Li X**, **Wu T-T**, **Sun R**. 2016. Coupling high-throughput genetics with phylogenetic information reveals an epistatic interaction on the influenza A virus M segment. BMC Genomics **17**:46.

56. **Raman R**, **Tharakaraman K**, **Shriver Z**, **Jayaraman A**, **Sasisekharan V**, **Sasisekharan R**. 2014. Glycan receptor specificity as a useful tool for characterization and surveillance of influenza A virus. Trends Microbiol **22**:632–641.

57. **Tharakaraman K**, **Raman R**, **Viswanathan K**, **Stebbins NW**, **Jayaraman A**, **Krishnan A**, **Sasisekharan V**, **Sasisekharan R**. 2013. Structural determinants for naturally evolving H5N1 hemagglutinin to switch its receptor specificity. Cell **153**:1475–1485.

58. **Gong LI**, **Suchard MA**, **Bloom JD**. 2013. Stability-mediated epistasis constrains the evolution of an influenza protein. Elife **2**:e00631.

59. **Russell CJ**. 2014. Acid-induced membrane fusion by the hemagglutinin protein and its role in influenza virus biology. Curr Top Microbiol Immunol **385**:93–116.

60. **Gog JR**, **Pellis L**, **Wood JLN**, **McLean AR**, **Arinaminpathy N**, **Lloyd-Smith JO**. 2015. Seven challenges in modeling pathogen dynamics within-host and across scales. Epidemics **10**:45–48.

61. **Park M**, **Loverdo C**, **Schreiber SJ**, **Lloyd-Smith JO**. 2013. Multiple scales of selection influence the evolutionary emergence of novel pathogens. Philosophical Transactions of the Royal Society B: Biological Sciences **368**:20120333–20120333.

62. **Beauchemin CAA**, **Handel A**. 2011. A review of mathematical models of influenza A infections within a host or cell culture: lessons learned and challenges ahead. BMC Public Health **11 Suppl 1**:S7.

63. **Lloyd-Smith JO**, **Funk S**, **McLean AR**, **Riley S**, **Wood JLN**. 2015. Nine challenges in modelling the emergence of novel pathogens. Epidemics **10**:35–39.

64. **Moncla LH**, **Zhong G**, **Nelson CW**, **Dinis JM**, **Mutschler J**, **Hughes AL**, **Watanabe T**, **Kawaoka Y**, **Friedrich TC**. 2016. Selective Bottlenecks Shape Evolutionary Pathways Taken during Mammalian Adaptation of a 1918-like Avian Influenza Virus. Cell Host Microbe **19**:169–180.

65. **Wilker PR**, **Dinis JM**, **Starrett G**, **Imai M**, **Hatta M**, **Nelson CW**, **O'Connor DH**, **Hughes AL**, **Neumann G**, **Kawaoka Y**, **Friedrich TC**. 2013. Selection on haemagglutinin imposes a bottleneck during mammalian transmission of reassortant H5N1 influenza viruses. Nat Commun **4**:2636.

66. **Zaraket H**, **Baranovich T**, **Kaplan BS**, **Carter R**, **Song M-S**, **Paulson JC**, **Rehg JE**, **Bahl J**, **Crumpton JC**, **Seiler J**, **Edmonson M**, **Wu G**, **Karlsson E**, **Fabrizio T**, **Zhu H**, **Guan Y**, **Husain M**, **Schultz-Cherry S**, **Krauss S**, **McBride R**, **Webster RG**, **Govorkova EA**, **Zhang J**, **Russell CJ**, **Webby RJ**. 2015. Mammalian adaptation of influenza A(H7N9) virus is limited by a narrow genetic bottleneck. Nat Commun **6**:6553.

67. **King G**, **Zeng L**. 2001. Logistic Regression in Rare Events Data. Political Analysis **9**:137–163.

68. **Hansson SO**. 2006. Economic (ir)rationality in risk analysis. Econ Phil **22**:231–241.

69. **Kucharski AJ**, **Mills HL**, **Donnelly CA**, **Riley S**. 2015. Transmission Potential of Influenza A(H7N9) Virus, China, 2013-2014. Emerging Infect Dis **21**:852–855.

70. **Russell RJ**, **Stevens DJ**, **Haire LF**, **Gamblin SJ**, **Skehel JJ**. 2006. Avian and human receptor binding by hemagglutinins of influenza A viruses. Glycoconj J **23**:85–92.

71. **Cauldwell AV**, **Long JS**, **Moncorgé O**, **Barclay WS**. 2014. Viral determinants of influenza A virus host range. J Gen Virol **95**:1193–1210.

72. **Naffakh N**, **Tomoiu A**, **Rameix-Welti M-A**, **van der Werf S**. 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. Annual review of microbiology **62**:403–424.

73. **Seladi-Schulman J**, **Campbell PJ**, **Suppiah S**, **Steel J**, **Lowen AC**. 2014. Filament-producing mutants of influenza A/Puerto Rico/8/1934 (H1N1) virus have higher neuraminidase activities than the spherical wild-type. PLoS ONE **9**:e112462.

74. **Seladi-Schulman J**, **Steel J**, **Lowen AC**. 2013. Spherical influenza viruses have a fitness advantage in embryonated eggs, while filament-producing strains are selected in vivo. J Virol **87**:13343–13353.

75. **Campbell PJ**, **Danzy S**, **Kyriakis CS**, **Deymier MJ**, **Lowen AC**, **Steel J**. 2014. The M segment of the 2009 pandemic influenza virus confers increased neuraminidase activity, filamentous morphology, and efficient contact transmissibility to A/Puerto Rico/8/1934-based reassortant viruses. J Virol **88**:3802–3814.

76. **Beale R**, **Wise H**, **Stuart A**, **Ravenhill BJ**, **Digard P**, **Randow F**. 2014. A LC3-interacting motif in the influenza A virus M2 protein is required to subvert autophagy and maintain virion stability. Cell Host Microbe **15**:239–247.

77. **Blumenkrantz D**, **Roberts KL**, **Shelton H**, **Lycett S**, **Barclay WS**. 2013. The short stalk length of highly pathogenic avian influenza H5N1 virus neuraminidase limits transmission of pandemic H1N1 virus in ferrets. J Virol **87**:10539–10551.

78. **Rajsbaum R**, **Albrecht RA**, **Wang MK**, **Maharaj NP**, **Versteeg GA**, **Nistal-Villán E**, **García-Sastre A**, **Gack MU**. 2012. Species-Specific Inhibition of RIG-I Ubiquitination and IFN Induction by the Influenza A Virus NS1 Protein. PLoS Pathog **8**:e1003059.

79. **Zanin M**, **Marathe B**, **Wong S-S**, **Yoon S-W**, **Collin E**, **Oshansky C**, **Jones J**, **Hause B**, **Webby R**. 2015. Pandemic Swine H1N1 Influenza Viruses with Almost Undetectable Neuraminidase Activity Are Not Transmitted via Aerosols in Ferrets and Are Inhibited by Human Mucus but Not Swine Mucus. J Virol **89**:5935–5948.

80. **Baum LG**, **Paulson JC**. 1991. The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. Virology **180**:10–15.

81. **Yen H-L**, **Liang C-H**, **Wu C-Y**, **Forrest HL**, **Ferguson A**, **Choy K-T**, **Jones J**, **Wong DD-Y**, **Cheung PP-H**, **Hsu C-H**, **Li OT**, **Yuen KM**, **Chan RWY**, **Poon LLM**, **Chan MCW**, **Nicholls JM**, **Krauss S**, **Wong C-H**, **Guan Y**, **Webster RG**, **Webby RJ**, **Peiris M**. 2011. Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proceedings of the National Academy of Sciences **108**:14264–14269.

82. **Handel A**, **Akin V**, **Pilyugin SS**, **Zarnitsyna V**, **Antia R**. 2014. How sticky should a virus be? The impact of virus binding and release on transmission fitness using influenza as an example. Journal of the Royal Society, Interface / the Royal Society **11**:20131083–20131083.

83. **Gambaryan AS**, **Tuzikov AB**, **Piskarev VE**, **Yamnikova SS**, **Lvov DK**, **Robertson JS**,

Bovin NV, **Matrosovich MN**. 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl(N-acetyllactosamine). Virology **232**:345–350.

84. **van Riel D**, **Munster VJ**, **de Wit E**, **Rimmelzwaan GF**, **Fouchier RAM**, **Osterhaus ADME**, **Kuiken T**. 2006. H5N1 Virus Attachment to Lower Respiratory Tract. Science **312**:399–399.

85. **Shinya K**, **Ebina M**, **Yamada S**, **Ono M**, **Kasai N**, **Kawaoka Y**. 2006. Avian flu: influenza virus receptors in the human airway. Nature **440**:435–436.

86. **Chandrasekaran A**, **Srinivasan A**, **Raman R**, **Viswanathan K**, **Raguram S**, **Tumpey TM**, **Sasisekharan V**, **Sasisekharan R**. 2008. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nature Biotechnology **26**:107–113.

87. **Lakdawala SS**, **Jayaraman A**, **Halpin RA**, **Lamirande EW**, **Shih AR**, **Stockwell TB**, **Lin X**, **Simenauer A**, **Hanson CT**, **Vogel L**, **Paskel M**, **Minai M**, **Moore I**, **Orandle M**, **Das SR**, **Wentworth DE**, **Sasisekharan R**, **Subbarao K**. 2015. The soft palate is an important site of adaptation for transmissible influenza viruses. Nature **526**:122–125.

88. **Koel BF**, **Burke DF**, **Bestebroer TM**, **van der Vliet S**, **Zondag GCM**, **Vervaet G**, **Skepner E**, **Lewis NS**, **Spronken MIJ**, **Russell CA**, **Eropkin MY**, **Hurt AC**, **Barr IG**, **de Jong JC**, **Rimmelzwaan GF**, **Osterhaus ADME**, **Fouchier RAM**, **Smith DJ**. 2013. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science **342**:976–979.

89. **Ha Y**, **Stevens DJ**, **Skehel JJ**, **Wiley DC**. 2001. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc Natl Acad Sci USA **98**:11181–11186.

90. **Ha Y**, **Stevens DJ**, **Skehel JJ**, **Wiley DC**. 2003. X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus. Virology **309**:209–218.

91. **Gamblin SJ**, **Haire LF**, **Russell RJ**, **Stevens DJ**, **Xiao B**, **Ha Y**, **Vasisht N**, **Steinhauer DA**, **Daniels RS**, **Elliot A**, **Wiley DC**, **Skehel JJ**. 2004. The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science **303**:1838–1842.

92. **Liu J**, **Stevens DJ**, **Haire LF**, **Walker PA**, **Coombs PJ**, **Russell RJ**, **Gamblin SJ**, **Skehel JJ**. 2009. Structures of receptor complexes formed by hemagglutinins

from the Asian Influenza pandemic of 1957. Proceedings of the National Academy of Sciences **106**:17175–17180.

93. **Xu R**, **McBride R**, **Paulson JC**, **Basler CF**, **Wilson IA**. 2010. Structure, receptor binding, and antigenicity of influenza virus hemagglutinins from the 1957 H2N2 pandemic. J Virol **84**:1715–1721.

94. **Yang H**, **Chen L-M**, **Carney PJ**, **Donis RO**, **Stevens J**. 2010. Structures of receptor complexes of a North American H7N2 influenza hemagglutinin with a loop deletion in the receptor binding site. PLoS Pathog **6**:e1001081.

95. **Lin YP**, **Xiong X**, **Wharton SA**, **Martin SR**, **Coombs PJ**, **Vachieri SG**, **Christodoulou E**, **Walker PA**, **Liu J**, **Skehel JJ**, **Gamblin SJ**, **Hay AJ**, **Daniels RS**, **McCauley JW**. 2012. Evolution of the receptor binding properties of the influenza A(H3N2) hemagglutinin. Proceedings of the National Academy of Sciences **109**:21474–21479.

96. **Yang H**, **Carney PJ**, **Donis RO**, **Stevens J**. 2012. Structure and receptor complexes of the hemagglutinin from a highly pathogenic H7N7 influenza virus. J Virol **86**:8645–8652.

97. **Zhang W**, **Shi Y**, **Qi J**, **Gao F**, **Li Q**, **Fan Z**, **Yan J**, **Gao GF**. 2013. Molecular basis of the receptor binding specificity switch of the hemagglutinins from both the 1918 and 2009 pandemic influenza A viruses by a D225G substitution. J Virol **87**:5949–5958.

98. **Matrosovich M**, **Tuzikov A**, **Bovin N**, **Gambaryan A**, **Klimov A**, **Castrucci MR**, **Donatelli I**, **Kawaoka Y**. 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol **74**:8502–8512.

99. **Chutinimitkul S**, **van Riel D**, **Munster VJ**, **van den Brand JMA**, **Rimmelzwaan GF**, **Kuiken T**, **Osterhaus ADME**, **Fouchier RAM**, **de Wit E**. 2010. In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity. J Virol **84**:6825–6833.

100. **Tumpey TM**, **Maines TR**, **Van Hoeven N**, **Glaser L**, **Solórzano A**, **Pappas C**, **Cox NJ**, **Swayne DE**, **Palese P**, **Katz JM**, **García-Sastre A**. 2007. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science **315**:655–659.

101. **Paulson JC**, **de Vries RP**. 2013. H5N1 receptor specificity as a factor in pandemic risk. Virus Res **178**:99–113.

102. **Xu D**, **Newhouse EI**, **Amaro RE**, **Pao HC**, **Cheng LS**, **Markwick PRL**, **McCammon JA**, **Li WW**, **Arzberger PW**. 2009. Distinct glycan topology for avian and human

sialopentasaccharide receptor analogues upon binding different hemagglutinins: a molecular dynamics perspective. Journal of Molecular Biology **387**:465–491.

103. **Paulson JC**, **Rogers GN**. 1987. Resialylated erythrocytes for assessment of the specificity of sialyloligosaccharide binding proteins. Meth Enzymol **138**:162–168.

104. **Ito T**, **Suzuki Y**, **Mitnaul L**, **Vines A**, **Kida H**, **Kawaoka Y**. 1997. Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology **227**:493–499.

105. **Gambaryan A**, **Tuzikov A**, **Pazynina G**, **Bovin N**, **Balish A**, **Klimov A**. 2006. Evolution of the receptor binding phenotype of influenza A (H5) viruses. Virology **344**:432–438.

106. **Gambaryan AS**, **Matrosovich MN**. 1992. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. J Virol Methods **39**:111–123.

107. **Xiong X**, **Coombs PJ**, **Martin SR**, **Liu J**, **Xiao H**, **McCauley JW**, **Locher K**, **Walker PA**, **Collins PJ**, **Kawaoka Y**, **Skehel JJ**, **Gamblin SJ**. 2013. Receptor binding by a ferret-transmissible H5 avian influenza virus. Nature **497**:392–396.

108. **Blixt O**, **Head S**, **Mondala T**, **Scanlan C**, **Huflejt ME**, **Alvarez R**, **Bryan MC**, **Fazio F**, **Calarese D**, **Stevens J**, **Razi N**, **Stevens DJ**, **Skehel JJ**, **van Die I**, **Burton DR**, **Wilson IA**, **Cummings R**, **Bovin N**, **Wong C-H**, **Paulson JC**. 2004. Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc Natl Acad Sci USA **101**:17033–17038.

109. **Childs RA**, **Palma AS**, **Wharton S**, **Matrosovich T**, **Liu Y**, **Chai W**, **Campanero-Rhodes MA**, **Zhang Y**, **Eickmann M**, **Kiso M**, **Hay A**, **Matrosovich M**, **Feizi T**. 2009. Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. Nature Biotechnology **27**:797–799.

110. **Stevens J**, **Blixt O**, **Paulson JC**, **Wilson IA**. 2006. Glycan microarray technologies: tools to survey host specificity of influenza viruses. Nat Rev Microbiol **4**:857–864.

111. **Walther T**, **Karamanska R**, **Chan RWY**, **Chan MCW**, **Jia N**, **Air G**, **Hopton C**, **Wong MP**, **Dell A**, **Malik Peiris JS**, **Haslam SM**, **Nicholls JM**. 2013. Glycomic analysis of human respiratory tract tissues and correlation with influenza virus infection. PLoS Pathog **9**:e1003223.

112. **Chan MCW**, **Chan RWY**, **Chan LLY**, **Mok CKP**, **Hui KPY**, **Fong JHM**, **Tao KP**, **Poon LLM**, **Nicholls JM**, **Guan Y**, **Peiris JSM**. 2013. Tropism and innate host responses of a novel avian influenza A H7N9 virus: an analysis of ex-vivo and in-vitro cultures of the human respiratory tract. Lancet Respir Med **1**:534–542.

113. **Chen L-M**, **Blixt O**, **Stevens J**, **Lipatov AS**, **Davis CT**, **Collins BE**, **Cox NJ**, **Paulson JC**, **Donis RO**. 2012. In vitro evolution of H5N1 avian influenza virus toward human-type receptor specificity. Virology **422**:105–113.

114. **Leigh MW**, **Connor RJ**, **Kelm S**, **Baum LG**, **Paulson JC**. 1995. Receptor specificity of influenza virus influences severity of illness in ferrets. Vaccine **13**:1468–1473.

115. **Shriver Z**, **Raman R**, **Viswanathan K**, **Sasisekharan R**. 2009. Context-specific target definition in influenza a virus hemagglutinin-glycan receptor interactions. Chem Biol **16**:803–814.

116. **Takemoto DK**, **Skehel JJ**, **Wiley DC**. 1996. A surface plasmon resonance assay for the binding of influenza virus hemagglutinin to its sialic acid receptor. Virology **217**:452–458.

117. **Collins BE**, **Paulson JC**. 2004. Cell surface biology mediated by low affinity multivalent protein-glycan interactions. Curr Opin Chem Biol **8**:617–625.

118. **Chen L-M**, **Rivailler P**, **Hossain J**, **Carney P**, **Balish A**, **Perry I**, **Davis CT**, **Garten R**, **Shu B**, **Xu X**, **Klimov A**, **Paulson JC**, **Cox NJ**, **Swenson S**, **Stevens J**, **Vincent A**, **Gramer M**, **Donis RO**. 2011. Receptor specificity of subtype H1 influenza A viruses isolated from swine and humans in the United States. Virology **412**:401–410.

119. **Stevens J**, **Chen L-M**, **Carney PJ**, **Garten R**, **Foust A**, **Le J**, **Pokorny BA**, **Manojkumar R**, **Silverman J**, **Devis R**, **Rhea K**, **Xu X**, **Bucher DJ**, **Paulson JC**, **Paulson J**, **Cox NJ**, **Klimov A**, **Donis RO**. 2010. Receptor specificity of influenza A H3N2 viruses isolated in mammalian cells and embryonated chicken eggs. J Virol **84**:8287–8299.

120. **Yamada S**, **Suzuki Y**, **Suzuki T**, **Le MQ**, **Nidom CA**, **Sakai-Tagawa Y**, **Muramoto Y**, **Ito M**, **Kiso M**, **Horimoto T**, **Shinya K**, **Sawada T**, **Kiso M**, **Usui T**, **Murata T**, **Lin Y**, **Hay A**, **Haire LF**, **Stevens DJ**, **Russell RJ**, **Gamblin SJ**, **Skehel JJ**, **Kawaoka Y**. 2006. Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature **444**:378–382.

121. **Li Q**, **Wang X**, **Gao Z**, **Sun Z**, **Cui Z**, **Duan Z**, **Li J**, **Gu M**, **Wang X**, **Hu J**, **Liu X**, **Liu X**. 2015. Novel reassortant H5N5 viruses bind to a human-type receptor as a factor in pandemic risk. Vet Microbiol **175**:356–361.

122. **Belser JA**, **Blixt O**, **Chen L-M**, **Pappas C**, **Maines TR**, **Van Hoeven N**, **Donis R**, **Busch J**, **McBride R**, **Paulson JC**, **Katz JM**, **Tumpey TM**. 2008. Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. Proceedings of the National Academy of Sciences **105**:7558–7563.

123. **Schrauwen EJA**, **Richard M**, **Burke DF**, **Rimmelzwaan GF**, **Herfst S**, **Fouchier RAM**. 2016. Amino Acid Substitutions That Affect Receptor Binding and Stability of the Hemagglutinin of Influenza A/H7N9 Virus. J Virol **90**:3794–3799.

124. **Matrosovich MN**, **Krauss S**, **Webster RG**. 2001. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology **281**:156–162.

125. **Li X**, **Shi J**, **Guo J**, **Deng G**, **Zhang Q**, **Wang J**, **He X**, **Wang K**, **Chen J**, **Li Y**, **Fan J**, **Kong H**, **Gu C**, **Guan Y**, **Suzuki Y**, **Kawaoka Y**, **Liu L**, **Jiang Y**, **Tian G**, **Li Y**, **Bu Z**, **Chen H**. 2014. Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 Avian Influenza viruses. PLoS Pathog **10**:e1004508.

126. **Xiong X**, **Martin SR**, **Haire LF**, **Wharton SA**, **Daniels RS**, **Bennett MS**, **McCauley JW**, **Collins PJ**, **Walker PA**, **Skehel JJ**, **Gamblin SJ**. 2013. Receptor binding by an H7N9 influenza virus from humans. Nature **499**:496–499.

127. **Di Lella S**, **Herrmann A**, **Mair CM**. 2016. Modulation of the pH Stability of Influenza Virus Hemagglutinin: A Host Cell Adaptation Strategy. Biophys J **110**:2293–2301.

128. **Zaraket H**, **Bridges OA**, **Duan S**, **Baranovich T**, **Yoon S-W**, **Reed ML**, **Salomon R**, **Webby RJ**, **Webster RG**, **Russell CJ**. 2013. Increased acid stability of the hemagglutinin protein enhances H5N1 influenza virus growth in the upper respiratory tract but is insufficient for transmission in ferrets. J Virol **87**:9911–9922.

129. **Skehel JJ**, **Wiley DC**. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem **69**:531–569.

130. **Mellman I**, **Fuchs R**, **Helenius A**. 1986. Acidification of the endocytic and exocytic pathways. Annu Rev Biochem **55**:663–700.

131. **Galloway SE**, **Reed ML**, **Russell CJ**, **Steinhauer DA**. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. PLoS Pathog **9**:e1003151.

132. **Cotter CR**, **Jin H**, **Chen Z**. 2014. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. PLoS Pathog **10**:e1003831.

133. **Maurer-Stroh S**, **Lee RTC**, **Eisenhaber F**, **Cui L**, **Phuah SP**, **Lin RT**. 2010. A new common mutation in the hemagglutinin of the 2009 (H1N1) influenza A virus. PLoS Curr **2**:RRN1162.

134. **Russier M**, **Yang G**, **Rehg JE**, **Wong S-S**, **Mostafa HH**, **Fabrizio TP**, **Barman S**,

Krauss S, **Webster RG**, **Webby RJ**, **Russell CJ**. 2016. Molecular requirements for a pandemic influenza virus: An acid-stable hemagglutinin protein. Proceedings of the National Academy of Sciences **113**:201524384–1641.

135. **Scholtissek C**. 1985. Stability of infectious influenza A viruses at low pH and at elevated temperature. Vaccine **3**:215–218.

136. **DuBois RM**, **Zaraket H**, **Reddivari M**, **Heath RJ**, **White SW**, **Russell CJ**. 2011. Acid stability of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity. PLoS Pathog **7**:e1002398.

137. **Reed ML**, **Yen H-L**, **DuBois RM**, **Bridges OA**, **Salomon R**, **Webster RG**, **Russell CJ**. 2009. Amino acid residues in the fusion peptide pocket regulate the pH of activation of the H5N1 influenza virus hemagglutinin protein. J Virol **83**:3568–3580.

138. **Zaraket H**, **Bridges OA**, **Russell CJ**. 2013. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus replication and pathogenesis in mice. J Virol **87**:4826–4834.

139. **Shelton H**, **Roberts KL**, **Molesti E**, **Temperton N**, **Barclay WS**. 2013. Mutations in haemagglutinin that affect receptor binding and pH stability increase replication of a PR8 influenza virus with H5 HA in the upper respiratory tract of ferrets and may contribute to transmissibility. J Gen Virol **94**:1220–1229.

140. **Mair CM**, **Ludwig K**, **Herrmann A**, **Sieben C**. 2014. Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection. Biochim Biophys Acta **1838**:1153–1168.

141. **Bullough PA**, **Hughson FM**, **Skehel JJ**, **Wiley DC**. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. Nature **371**:37–43.

142. **Wilson IA**, **Skehel JJ**, **Wiley DC**. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature **289**:366–373.

143. **Weis WI**, **Cusack SC**, **Brown JH**, **Daniels RS**, **Skehel JJ**, **Wiley DC**. 1990. The structure of a membrane fusion mutant of the influenza virus haemagglutinin. EMBO J **9**:17–24.

144. **de Vries RP**, **Zhu X**, **McBride R**, **Rigter A**, **Hanson A**, **Zhong G**, **Hatta M**, **Xu R**, **Yu W**, **Kawaoka Y**, **de Haan CAM**, **Wilson IA**, **Paulson JC**. 2014. Hemagglutinin receptor specificity and structural analyses of respiratory droplet-transmissible H5N1 viruses. J Virol **88**:768–773.

145. **Huang RT**, **Rott R**, **Wahn K**, **Klenk HD**, **Kohama T**. 1980. The function of the

neuraminidase in membrane fusion induced by myxoviruses. Virology **107**:313–319.

146. **Su B**, **Wurtzer S**, **Rameix-Welti M-A**, **Dwyer D**, **van der Werf S**, **Naffakh N**, **Clavel F**, **Labrosse B**. 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS ONE **4**:e8495.

147. **Reed ML**, **Bridges OA**, **Seiler P**, **Kim J-K**, **Yen H-L**, **Salomon R**, **Govorkova EA**, **Webster RG**, **Russell CJ**. 2010. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks. J Virol **84**:1527–1535.

148. **O'Donnell CD**, **Vogel L**, **Matsuoka Y**, **Jin H**, **Subbarao K**. 2014. The matrix gene segment destabilizes the acid and thermal stability of the hemagglutinin of pandemic live attenuated influenza virus vaccines. J Virol **88**:12374–12384.

149. **Hamilton BS**, **Whittaker GR**, **Daniel S**. 2012. Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion. Viruses **4**:1144–1168.

150. **Hoekstra D**, **de Boer T**, **Klappe K**, **Wilschut J**. 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry **23**:5675–5681.

151. **Loyter A**, **Citovsky V**, **Blumenthal R**. 1988. The use of fluorescence dequenching measurements to follow viral membrane fusion events. Methods Biochem Anal **33**:129–164.

152. **Hoekstra D**, **Klappe K**. 1993. Fluorescence assays to monitor fusion of enveloped viruses. Meth Enzymol **220**:261–276.

153. **Steinhauer DA**, **Martín J**, **Lin YP**, **Wharton SA**, **Oldstone MB**, **Skehel JJ**, **Wiley DC**. 1996. Studies using double mutants of the conformational transitions in influenza hemagglutinin required for its membrane fusion activity. Proc Natl Acad Sci USA **93**:12873–12878.

154. **Ruigrok RW**, **Martin SR**, **Wharton SA**, **Skehel JJ**, **Bayley PM**, **Wiley DC**. 1986. Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. Virology **155**:484–497.

155. **Carr CM**, **Chaudhry C**, **Kim PS**. 1997. Influenza hemagglutinin is spring-loaded by a metastable native conformation. Proc Natl Acad Sci USA **94**:14306–14313.

156. **Huang TS**, **Palese P**, **Krystal M**. 1990. Determination of influenza virus proteins required for genome replication. J Virol **64**:5669–5673.

157. **Spooner LL**, **Barry RD**. 1977. Participation of DNA-dependent RNA polymerase II in replication of influenza viruses. Nature **268**:650–652.

158. **Subbarao EK**, **London W**, **Murphy BR**. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol **67**:1761–1764.

159. **Mehle A**, **Doudna JA**. 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. Proceedings of the National Academy of Sciences **106**:21312–21316.

160. **Long JS**, **Giotis ES**, **Moncorgé O**, **Frise R**, **Mistry B**, **James J**, **Morisson M**, **Iqbal M**, **Vignal A**, **Skinner MA**, **Barclay WS**. 2016. Species difference in ANP32A underlies influenza A virus polymerase host restriction. Nature **529**:101–104.

161. **Resa-Infante P**, **Jorba N**, **Zamarreño N**, **Fernández Y**, **Juárez S**, **Ortín J**. 2008. The host-dependent interaction of alpha-importins with influenza PB2 polymerase subunit is required for virus RNA replication. PLoS ONE **3**:e3904.

162. **Gabriel G**, **Dauber B**, **Wolff T**, **Planz O**, **Klenk HD**, **Stech J**. 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci USA **102**:18590–18595.

163. **Sediri H**, **Schwalm F**, **Gabriel G**, **Klenk H-D**. 2015. Adaptive mutation PB2 D701N promotes nuclear import of influenza vRNPs in mammalian cells. Eur J Cell Biol **94**:368–374.

164. **de Jong RMC**, **Stockhofe-Zurwieden N**, **Verheij ES**, **de Boer-Luijtze EA**, **Ruiter SJM**, **de Leeuw OS**, **Cornelissen LAHM**. 2013. Rapid emergence of a virulent PB2 E627K variant during adaptation of highly pathogenic avian influenza H7N7 virus to mice. Virol J **10**:276.

165. **Long JS**, **Howard WA**, **Núñez A**, **Moncorgé O**, **Lycett S**, **Banks J**, **Barclay WS**. 2013. The effect of the PB2 mutation 627K on highly pathogenic H5N1 avian influenza virus is dependent on the virus lineage. J Virol **87**:9983–9996.

166. **Moncorgé O**, **Mura M**, **Barclay WS**. 2010. Evidence for avian and human host cell factors that affect the activity of influenza virus polymerase. J Virol **84**:9978–9986.

167. **Robb NC**, **Smith M**, **Vreede FT**, **Fodor E**. 2009. NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome. J Gen Virol **90**:1398–1407.

168. **Mänz B**, **Brunotte L**, **Reuther P**, **Schwemmle M**. 2012. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. Nat Commun **3**:802.

169. **Chua MA**, **Schmid S**, **Perez JT**, **Langlois RA**, **tenOever BR**. 2013. Influenza A virus utilizes suboptimal splicing to coordinate the timing of infection. Cell Rep **3**:23–29.

170. **Paterson D**, **Fodor E**. 2012. Emerging roles for the influenza A virus nuclear export protein (NEP). PLoS Pathog **8**:e1003019.

171. **Robb NC**, **Fodor E**. 2012. The accumulation of influenza A virus segment 7 spliced mRNAs is regulated by the NS1 protein. J Gen Virol **93**:113–118.

172. **Herfst S**, **Chutinimitkul S**, **Ye J**, **de Wit E**, **Munster VJ**, **Schrauwen EJA**, **Bestebroer TM**, **Jonges M**, **Meijer A**, **Koopmans M**, **Rimmelzwaan GF**, **Osterhaus ADME**, **Perez DR**, **Fouchier RAM**. 2010. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. J Virol **84**:3752–3758.

173. **Zhang R**, **Chen T**, **Ou X**, **Liu R**, **Yang Y**, **Ye W**, **Chen J**, **Yao D**, **Sun B**, **Zhang X**, **Zhou J**, **Sun Y**, **Chen F**, **Wang S-P**. 2016. Clinical, epidemiological and virological characteristics of the first detected human case of avian influenza A(H5N6) virus. Infect Genet Evol **40**:236–242.

174. **Reich S**, **Guilligay D**, **Pflug A**, **Malet H**, **Berger I**, **Crépin T**, **Hart D**, **Lunardi T**, **Nanao M**, **Ruigrok RWH**, **Cusack S**. 2014. Structural insight into cap-snatching and RNA synthesis by influenza polymerase. Nature **516**:361–366.

175. **Krieger E**, **Vriend G**. 2014. YASARA View - molecular graphics for all devices - from smartphones to workstations. Bioinformatics **30**:2981–2982.

176. **Jagger BW**, **Memoli MJ**, **Sheng Z-M**, **Qi L**, **Hrabal RJ**, **Allen GL**, **Dugan VG**, **Wang R**, **Digard P**, **Kash JC**, **Taubenberger JK**. 2010. The PB2-E627K mutation attenuates viruses containing the 2009 H1N1 influenza pandemic polymerase. MBio **1**.

177. **Yamada S**, **Hatta M**, **Staker BL**, **Watanabe S**, **Imai M**, **Shinya K**, **Sakai-Tagawa Y**, **Ito M**, **Ozawa M**, **Watanabe T**, **Sakabe S**, **Li C**, **Kim JH**, **Myler PJ**, **Phan I**, **Raymond A**, **Smith E**, **Stacy R**, **Nidom CA**, **Lank SM**, **Wiseman RW**, **Bimber BN**, **O'Connor DH**, **Neumann G**, **Stewart LJ**, **Kawaoka Y**. 2010. Biological and structural characterization of a host-adapting amino acid in influenza virus. PLoS Pathog **6**:e1001034.

178. **Holder BP**, **Simon P**, **Liao LE**, **Abed Y**, **Bouhy X**, **Beauchemin CAA**, **Boivin G**. 2011. Assessing the in vitro fitness of an oseltamivir-resistant seasonal A/H1N1 influenza strain using a mathematical model. PLoS ONE **6**:e14767.

179. **Simon P**, **Holder BP**, **Bouhy X**, **Abed Y**, **Beauchemin CAA**, **Boivin G**. 2011. The I222V neuraminidase mutation has a compensatory role in replication of an

oseltamivir-resistant influenza virus A/H3N2 E119V mutant. J Clin Microbiol **49**:715–717.

180. **Hanson A**, **Imai M**, **Hatta M**, **McBride R**, **Imai H**, **Taft A**, **Zhong G**, **Watanabe T**, **Suzuki Y**, **Neumann G**, **Paulson JC**, **Kawaoka Y**. 2015. Identification of Stabilizing Mutations in an H5 Hemagglutinin Influenza Virus Protein. J Virol **90**:2981–2992.

181. **Thyagarajan B**, **Bloom JD**. 2014. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. Elife **3**:R119.

182. **Lipsitch M**, **Inglesby TV**. 2014. Moratorium on Research Intended To Create Novel Potential Pandemic Pathogens. MBio **5**:e02366–14–e02366–14.

183. **Belser JA**, **Maines TR**, **Katz JM**, **Tumpey TM**. 2013. Considerations regarding appropriate sample size for conducting ferret transmission experiments. Future Microbiol **8**:961–965.

184. **Nishiura H**, **Yen H-L**, **Cowling BJ**. 2013. Sample size considerations for one-to-one animal transmission studies of the influenza A viruses. PLoS ONE **8**:e55358.

185. **Mills CE**, **Robins JM**, **Bergstrom CT**, **Lipsitch M**. 2006. Pandemic influenza: risk of multiple introductions and the need to prepare for them. PLoS Med **3**:e135.

186. **Lipsitch M**, **Inglesby TV**. 2015. Reply to "Studies on influenza virus transmission between ferrets: the public health risks revisited". MBio **6**:e00041–15.

187. **Fouchier RAM**. 2015. Studies on influenza virus transmission between ferrets: the public health risks revisited. MBio **6**:e02560–14.

188. **Krenn BM**, **Egorov A**, **Romanovskaya-Romanko E**, **Wolschek M**, **Nakowitsch S**, **Ruthsatz T**, **Kiefmann B**, **Morokutti A**, **Humer J**, **Geiler J**, **Cinatl J**, **Michaelis M**, **Wressnigg N**, **Sturlan S**, **Ferko B**, **Batishchev OV**, **Indenbom AV**, **Zhu R**, **Kastner M**, **Hinterdorfer P**, **Kiselev O**, **Muster T**, **Romanova J**. 2011. Single HA2 mutation increases the infectivity and immunogenicity of a live attenuated H5N1 intranasal influenza vaccine candidate lacking NS1. PLoS ONE **6**:e18577.

189. **Bush RM**, **Smith CB**, **Cox NJ**, **Fitch WM**. 2000. Effects of passage history and sampling bias on phylogenetic reconstruction of human influenza A evolution. Proc Natl Acad Sci USA **97**:6974–6980.

190. **Danzig R**. 2011. Driving in the Dark. Center for a New American Security.

191. **Lloyd-Smith JO**, **Schreiber SJ**, **Kopp PE**, **Getz WM**. 2005. Superspreading and the effect of individual variation on disease emergence. Nature **438**:355–359.

192. **Lipsitch M**, **Cohen T**, **Cooper B**, **Robins JM**, **Ma S**, **James L**, **Gopalakrishna G**,

Chew SK, **Tan CC**, **Samore MH**, **Fisman D**, **Murray M**. 2003. Transmission dynamics and control of severe acute respiratory syndrome. Science **300**:1966–1970.

193. **Ferguson NM**, **Cummings DAT**, **Fraser C**, **Cajka JC**, **Cooley PC**, **Burke DS**. 2006. Strategies for mitigating an influenza pandemic. Nature **442**:448–452.

194. **Merler S**, **Ajelli M**, **Fumanelli L**, **Vespignani A**. 2013. Containing the accidental laboratory escape of potential pandemic influenza viruses. BMC Med **11**:252.

195. **Cooper BS**, **Pitman RJ**, **Edmunds WJ**, **Gay NJ**. 2006. Delaying the international spread of pandemic influenza. PLoS Med **3**:e212.

196. **Smith GJD**, **Donis RO**, **World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5 Evolution Working Group**. 2015. Nomenclature updates resulting from the evolution of avian influenza A(H5) virus clades 2.1.3.2a, 2.2.1, and 2.3.4 during 2013-2014. Influenza Other Respi Viruses **9**:271–276.

197. **WHO/OIE/FAO H5N1 Evolution Working Group**. 2008. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerging Infect Dis **14**:e1–e1.

198. **Hill AA**, **Dewé T**, **Kosmider R**, **Dobschuetz Von S**, **Munoz O**, **Hanna A**, **Fusaro A**, **De Nardi M**, **Howard W**, **Stevens K**, **Kelly L**, **Havelaar A**, **Stärk K**. 2015. Modelling the species jump: towards assessing the risk of human infection from novel avian influenzas. R Soc Open Sci **2**:150173.

199. **Gilbert M**, **Golding N**, **Zhou H**, **Wint GRW**, **Robinson TP**, **Tatem AJ**, **Lai S**, **Zhou S**, **Jiang H**, **Guo D**, **Huang Z**, **Messina JP**, **Xiao X**, **Linard C**, **Van Boeckel TP**, **Martin V**, **Bhatt S**, **Gething PW**, **Farrar JJ**, **Hay SI**, **Yu H**. 2014. Predicting the risk of avian influenza A H7N9 infection in live-poultry markets across Asia. Nat Commun **5**:4116.

200. **Nelson MI**, **Stratton J**, **Killian ML**, **Janas-Martindale A**, **Vincent AL**. 2015. Continual Reintroduction of Human Pandemic H1N1 Influenza A Viruses into Swine in the United States, 2009 to 2014. J Virol **89**:6218–6226.

201. **Nelson MI**, **Wentworth DE**, **Culhane MR**, **Vincent AL**, **Viboud C**, **LaPointe MP**, **Lin X**, **Holmes EC**, **Detmer SE**. 2014. Introductions and evolution of human-origin seasonal influenza a viruses in multinational swine populations. J Virol **88**:10110–10119.

202. **Lewis NS**, **Russell CA**, **Langat P**, **Anderson TK**, **Berger K**, **Bielejec F**, **Burke DF**, **Dudas G**, **Fonville JM**, **Fouchier RA**, **Kellam P**, **Koel BF**, **Lemey P**, **Nguyen T**, **Nuansrichy B**, **Peiris JM**, **Saito T**, **Simon G**, **Skepner E**, **Takemae N**, **ESNIP3**

consortium, **Webby RJ**, **Van Reeth K**, **Brookes SM**, **Larsen L**, **Watson SJ**, **Brown IH**, **Vincent AL**. 2016. The global antigenic diversity of swine influenza A viruses. Elife **5**:e01914.

203. **Na W**, **Lyoo K-S**, **Song E-J**, **Hong M**, **Yeom M**, **Moon H**, **Kang B-K**, **Kim D-J**, **Kim J-K**, **Song D**. 2015. Viral dominance of reassortants between canine influenza H3N2 and pandemic (2009) H1N1 viruses from a naturally co-infected dog. Virol J **12**:134.

204. **Flanagan ML**, **Parrish CR**, **Cobey S**, **Glass GE**, **Bush RM**, **Leighton TJ**. 2012. Anticipating the species jump: surveillance for emerging viral threats. Zoonoses Public Health **59**:155–163.

205. **Meyer AG**, **Wilke CO**. 2015. Geometric Constraints Dominate the Antigenic Evolution of Influenza H3N2 Hemagglutinin. PLoS Pathog **11**:e1004940.

206. **Meyer AG**, **Dawson ET**, **Wilke CO**. 2013. Cross-species comparison of site-specific evolutionary-rate variation in influenza haemagglutinin. Philosophical Transactions of the Royal Society B: Biological Sciences **368**:20120334.

207. **Tusche C**, **Steinbrück L**, **McHardy AC**. 2012. Detecting patches of protein sites of influenza A viruses under positive selection. Mol Biol Evol **29**:2063–2071.

208. **Kratsch C**, **Klingen TR**, **Mümken L**, **Steinbrück L**, **McHardy AC**. 2016. Determination of antigenicity-altering patches on the major surface protein of human influenza A/H3N2 viruses. Virus Evolution **2**:vev025.

209. **McWhite CD**, **Meyer AG**, **Wilke CO**. 2016. Sequence amplification via cell passaging creates spurious signals of positive adaptation in influenza virus H3N2 hemagglutinin. Virus Evolution **2**:vew026–14.

210. **Kasson PM**, **Ensign DL**, **Pande VS**. 2009. Combining molecular dynamics with bayesian analysis to predict and evaluate ligand-binding mutations in influenza hemagglutinin. J Am Chem Soc **131**:11338–11340.

211. **Kasson PM**, **Pande VS**. 2009. Combining mutual information with structural analysis to screen for functionally important residues in influenza hemagglutinin. Pac Symp Biocomput 492–503.

212. **Soundararajan V**, **Zheng S**, **Patel N**, **Warnock K**, **Raman R**, **Wilson IA**, **Raguram S**, **Sasisekharan V**, **Sasisekharan R**. 2011. Networks link antigenic and receptor-binding sites of influenza hemagglutinin: mechanistic insight into fitter strain propagation. Sci Rep **1**:200.

213. **Maurer-Stroh S**, **Li Y**, **Bastien N**, **Gunalan V**, **Lee RTC**, **Eisenhaber F**, **Booth TF**. 2014. Potential human adaptation mutation of influenza A(H5N1) virus, Canada.

Emerging Infect Dis **20**:1580–1582.

214. **Bergelson J**, **Roux F**. 2010. Towards identifying genes underlying ecologically relevant traits in Arabidopsis thaliana. Nat Rev Genet **11**:867–879.

215. **Leslie R**, **O'Donnell CJ**, **Johnson AD**. 2014. GRASP: analysis of genotype-phenotype results from 1390 genome-wide association studies and corresponding open access database. Bioinformatics **30**:i185–94.

216. **Ashenberg O**, **Gong LI**, **Bloom JD**. 2013. Mutational effects on stability are largely conserved during protein evolution. Proceedings of the National Academy of Sciences **110**:21071–21076.

217. **Pinilla LT**, **Holder BP**, **Abed Y**, **Boivin G**, **Beauchemin CAA**. 2012. The H275Y neuraminidase mutation of the pandemic A/H1N1 influenza virus lengthens the eclipse phase and reduces viral output of infected cells, potentially compromising fitness in ferrets. J Virol **86**:10651–10660.

218. **Hanahan D**, **Weinberg RA**. 2011. Hallmarks of cancer: the next generation. Cell **144**:646–674.

219. **Kandoth C**, **McLellan MD**, **Vandin F**, **Ye K**, **Niu B**, **Lu C**, **Xie M**, **Zhang Q**, **McMichael JF**, **Wyczalkowski MA**, **Leiserson MDM**, **Miller CA**, **Welch JS**, **Walter MJ**, **Wendl MC**, **Ley TJ**, **Wilson RK**, **Raphael BJ**, **Ding L**. 2013. Mutational landscape and significance across 12 major cancer types. Nature **502**:333–339.

220. **Ledford H**. 2015. End of cancer-genome project prompts rethink. Nature.

221. **Weinberg R**. 2010. Point: Hypotheses first. Nature **464**:678–678.

222. **Bloom JD**. 2015. Software for the analysis and visualization of deep mutational scanning data. BMC Bioinformatics **16**:168.

223. **Fowler DM**, **Fields S**. 2014. Deep mutational scanning: a new style of protein science. Nat Methods **11**:801–807.

224. **Jayaraman A**, **Chandrasekaran A**, **Viswanathan K**, **Raman R**, **Fox JG**, **Sasisekharan R**. 2012. Decoding the distribution of glycan receptors for human-adapted influenza A viruses in ferret respiratory tract. PLoS ONE **7**:e27517.

225. **Jia N**, **Barclay WS**, **Roberts K**, **Yen H-L**, **Chan RWY**, **Lam AKY**, **Air G**, **Peiris JSM**, **Dell A**, **Nicholls JM**, **Haslam SM**. 2014. Glycomic characterization of respiratory tract tissues of ferrets: implications for its use in influenza virus infection studies. J Biol Chem **289**:28489–28504.

226. **Ng PSK**, **Böhm R**, **Hartley-Tassell LE**, **Steen JA**, **Wang H**, **Lukowski SW**,

Hawthorne PL, **Trezise AEO**, **Coloe PJ**, **Grimmond SM**, **Haselhorst T**, **Itzstein von M**, **Paton AW**, **Paton JC**, **Jennings MP**. 2014. Ferrets exclusively synthesize Neu5Ac and express naturally humanized influenza A virus receptors. Nat Commun **5**:5750.

227. **Belser JA**, **Szretter KJ**, **Katz JM**, **Tumpey TM**. 2009. Use of animal models to understand the pandemic potential of highly pathogenic avian influenza viruses. Adv Virus Res **73**:55–97.

228. **Peng X**, **Alföldi J**, **Gori K**, **Eisfeld AJ**, **Tyler SR**, **Tisoncik-Go J**, **Brawand D**, **Law GL**, **Skunca N**, **Hatta M**, **Gasper DJ**, **Kelly SM**, **Chang J**, **Thomas MJ**, **Johnson J**, **Berlin AM**, **Lara M**, **Russell P**, **Swofford R**, **Turner-Maier J**, **Young S**, **Hourlier T**, **Aken B**, **Searle S**, **Sun X**, **Yi Y**, **Suresh M**, **Tumpey TM**, **Siepel A**, **Wisely SM**, **Dessimoz C**, **Kawaoka Y**, **Birren BW**, **Lindblad-Toh K**, **Di Palma F**, **Engelhardt JF**, **Palermo RE**, **Katze MG**. 2014. The draft genome sequence of the ferret (Mustela putorius furo) facilitates study of human respiratory disease. Nature Biotechnology **32**:1250–1255.

229. **Andrews SF**, **Huang Y**, **Kaur K**, **Popova LI**, **Ho IY**, **Pauli NT**, **Henry Dunand CJ**, **Taylor WM**, **Lim S**, **Huang M**, **Qu X**, **Lee J-H**, **Salgado-Ferrer M**, **Krammer F**, **Palese P**, **Wrammert J**, **Ahmed R**, **Wilson PC**. 2015. Immune history profoundly affects broadly protective B cell responses to influenza. Science Translational Medicine **7**:316ra192–316ra192.

230. **Belser JA**, **Eckert AM**, **Tumpey TM**, **Maines TR**. 2016. Complexities in Ferret Influenza Virus Pathogenesis and Transmission Models. Microbiol Mol Biol Rev **80**:733–744.

231. **Lowen AC**, **Palese P**. 2007. Influenza virus transmission: basic science and implications for the use of antiviral drugs during a pandemic. Infect Disord Drug Targets **7**:318–328.

232. **Van Kerkhove MD**, **Mumford E**, **Mounts AW**, **Bresee J**, **Ly S**, **Bridges CB**, **Otte J**. 2011. Highly pathogenic avian influenza (H5N1): pathways of exposure at the animal-human interface, a systematic review. PLoS ONE **6**:e14582.

233. **Wang TT**, **Parides MK**, **Palese P**. 2012. Seroevidence for H5N1 influenza infections in humans: meta-analysis. Science **335**:1463–1463.

234. **Van Kerkhove MD**, **Riley S**, **Lipsitch M**, **Guan Y**, **Monto AS**, **Webster RG**, **Zambon M**, **Nicoll A**, **Peiris JSM**, **Ferguson NM**. 2012. Comment on "Seroevidence for H5N1 influenza infections in humans: meta-analysis". Science **336**:1506–author reply 1506.

235. **Chao D-Y**, **Cheng K-F**, **Li T-C**, **Wu T-N**, **Chen C-Y**, **Tsai C-A**, **Chen J-H**, **Chiu H-T**, **Lu**

J-J, **Su M-C**, **Liao Y-H**, **Chan W-C**, **Hsieh Y-H**. 2011. Serological Evidence of Subclinical Transmission of the 2009 Pandemic H1N1 Influenza Virus Outside of Mexico. PLoS ONE **6**:e14555.

236. **Huang SY**, **Yang JR**, **Lin YJ**, **Yang CH**, **CHENG MC**, **LIU MT**, **WU HS**, **CHANG FY**. 2015. Serological comparison of antibodies to avian influenza viruses, subtypes H5N2, H6N1, H7N3 and H7N9 between poultry workers and non-poultry workers in Taiwan in 2012. Epidemiol Infect **143**:2965–2974.

237. **Gomaa MR**, **Kayed AS**, **Elabd MA**, **Zeid DA**, **Zaki SA**, **Rifay El AS**, **Sherif LS**, **McKenzie PP**, **Webster RG**, **Webby RJ**, **Ali MA**, **Kayali G**. 2015. Avian influenza A(H5N1) and A(H9N2) seroprevalence and risk factors for infection among Egyptians: a prospective, controlled seroepidemiological study. J Infect Dis **211**:1399–1407.

238. **Kim JH**, **Lo FK**, **Cheuk KK**, **Kwong MS**, **Goggins WB**, **Cai YS**, **Lee SS**, **Griffiths S**. 2011. Knowledge of avian influenza (H5N1) among poultry workers, Hong Kong, China. Emerging Infect Dis **17**:2319–2321.

239. **Blair PJ**, **Putnam SD**, **Krueger WS**, **Chum C**, **Wierzba TF**, **Heil GL**, **Yasuda CY**, **Williams M**, **Kasper MR**, **Friary JA**, **Capuano AW**, **Saphonn V**, **Peiris M**, **Shao H**, **Perez DR**, **Gray GC**. 2013. Evidence for avian H9N2 influenza virus infections among rural villagers in Cambodia. Journal of Infection and Public Health **6**:69–79.

240. **Wang X**, **Fang S**, **Lu X**, **Xu C**, **Cowling BJ**, **Tang X**, **Peng B**, **Wu W**, **He J**, **Tang Y**, **Xie X**, **Mei S**, **Kong D**, **Zhang R**, **Ma H**, **Cheng J**. 2014. Seroprevalence to Avian Influenza A(H7N9) Virus Among Poultry Workers and the General Population in Southern China: A Longitudinal Study. Clin Infect Dis **59**:ciu399–e83.

241. **Yu X**, **Tsibane T**, **McGraw PA**, **House FS**, **Keefer CJ**, **Hicar MD**, **Tumpey TM**, **Pappas C**, **Perrone LA**, **Martinez O**, **Stevens J**, **Wilson IA**, **Aguilar PV**, **Altschuler EL**, **Basler CF**, **Crowe JE**. 2008. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature **455**:532–536.