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Sequence analysis and receptor specificity of the hemagglutinin of a recent influenza H2N2 virus isolated from chicken in North America

Laurel Glaser · Dmitriy Zamarin · Helen M. Acland · Erica Spackman · Peter Palese · Adolfo García-Sastre · Deepanker Tewari

Abstract Influenza viruses bind host cells following an interaction between the viral hemagglutinin (HA) protein and host cell sialylated glycoproteins and glycolipids. Differences in binding affinities of the HAs for different types of sialic acid linkages (α2-3 vs. α2-6) contribute to determining the host range of an influenza virus. The ability of an avian influenza virus HA to bind the human form of the receptor may be one requirement for an avian virus to propagate in the human population. In this paper, we describe the characterization of the HA from an H2N2 virus isolated from a Pennsylvania chicken farm in 2004. Sequence analysis revealed that this HA is a member of the Eurasian clade, and receptor binding studies show that it maintains its specificity for the avian influenza virus α2-3 linked sialic acid receptor.

L. Glaser · D. Zamarin
Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029, USA

H. M. Acland
Pennsylvania Veterinary Laboratory, Pennsylvania Department of Agriculture, Harrisburg, PA 17110, USA

E. Spackman
Southeast Poultry Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA 30605, USA

P. Palese · A. García-Sastre (✉)
Department of Microbiology and NorthEast Biodefense Center, Mount Sinai School of Medicine, New York, NY 10029, USA
e-mail: adolfo.garcia-sastre@mssm.edu

D. Tewari
Pennsylvania Veterinary Laboratory, Pennsylvania Department of Agriculture, Harrisburg, PA 17110, USA

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Abbreviations

A/ck/PA/2004	A/chicken/Pennsylvania/2004
BSA	bovine serum albumin
CRBC	chicken red blood cells
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
Gal	galactose
HA	hemagglutinin
GFP	green fluorescence protein
Gln	glutamine
Gly	glycine
NVSL	National Veterinary Services Laboratory
PBS	phosphate buffer saline
RT-PCR	reverse transcription-PCR
Sia	sialic acid
VCNA	*Vibrio cholerae* neuraminidase

Introduction

Influenza viruses cause yearly epidemics in the human population and less frequently, severe human pandemics characterized by excess mortality and morbidity. Usually harboring numerous subtypes of the virus without experiencing substantial illness, wild birds act as a natural reservoir for influenza A viruses [1]. In contrast, domestic poultry experience both virulent and avirulent influenza infections following adaptation of the virus from wild birds [2]. Human pandemics occur when re-assorted influenza viruses possessing avian surface glycoproteins against which humans have limited immunity infect and spread among people. However, pandemic influenza viruses arise only sporadically, with three

pandemic episodes known to have occurred in the last 100 years. One hypothesis to explain this phenomenon suggests that acquisition of critical mutations in the receptor binding protein, hemagglutinin (HA), is one of the requirements for an avian influenza virus to efficiently infect and propagate in humans and therefore to cross the species barrier from birds to humans.

The first step of influenza viral entry into the host cell involves binding of the viral surface glycoprotein HA to sialic acid on host cells. Sialic acid is a nine carbon sugar molecule that is usually attached to a galactose or *N*-acetylgalactosamine present in the sugar moieties of host cell glycoproteins or glycolipids. Although a specific glycoprotein or glycolipid receptor has not been identified, different influenza viruses have specific preferences for distinct forms of sialic acid. Specifically, viruses isolated from wild bird and most gallinaceous poultry sources prefer 5-*N*-acetylneuraminic acid attached to galactose by an α2-3 linkage (Siaα2-3Gal) and human viruses prefer 5-*N*-acetylneuraminic acid attached to galactose by an α2-6 linkage (Siaα2-6Gal) [3–7]. This observation correlates with the type of sialic acid expressed on host cell tissues usually infected by influenza virus. The human trachea contains predominately Siaα2-6Gal and the duck intestinal epithelium expresses mainly Siaα2-3Gal [8,9]. Isolated chicken epithelial cells express both Siaα2-6Gal and Siaα2-3Gal [10]. This evidence suggests that receptor binding specificity may be a host range determinant for influenza virus.

Evidence for the existence of selective pressure on receptor specificity depending on the host animal also exists. There are 16 recognized subtypes of HA that have conserved structural and functional features, but divergent sequences [11,12]. All subtypes infect birds, but only 3 subtypes, H1, H2 and H3, have caused sustained infections in the human population in the last century, having been responsible for the 1918, 1957 and 1968 pandemics, respectively. From 1957 to 1968, H2N2 influenza viruses circulated in humans, and genetically similar potential precursors were identified in birds [13–15]. Interestingly, some early human virus isolates bound Siaα2-6Gal with lower affinity than those isolated in the later years of the pandemic, suggesting the existence of a selective pressure on receptor binding [5]. After 1968, H3N2 viruses were introduced into humans coincident with the disappearance of H2N2 viruses from the human population. However, since the reintroduction of another H2 virus from birds to humans is always possible and all people born after 1968 are susceptible to an H2 virus due to the absence of previous exposure and pre-existing immunity to these viruses, it is important to understand the pandemic potential of H2 viruses currently circulating in the avian population.

Phylogenetic analysis of H2 HAs isolated as part of surveillance programs in wild birds between 1977–1998 revealed two different sublineages, American and Eurasian [16]. As the names imply, the sublineages reflect a geographical division. However, shorebirds from the US were found infected with H2 influenza viruses of the Eurasian lineage, suggesting some mixing. Here we describe the sequence and receptor binding specificity of an H2 HA recently isolated from a domestic chicken in Pennsylvania.

Isolation and antigenic characterization of influenza A/chicken/Pennsylvania/2004 (A/ck/PA/2004) virus

The influenza infection in chickens was first identified by detection of egg yolk antibody using agar gel diffusion test during routine farm monitoring (a layer bird operation) in Pennsylvania. Further testing of cloacal swab pool (5 swabs/sample pool) samples collected from the farm in viral transport medium consisting of Minimum Essential Medium containing gentamicin (200 μg/ml), kanamycin (50 U/ml), penicillin, streptomycin and amphotericin B combination (1%), and 15 mM HEPES buffer showed 2 of the 18 swab pools were positive by RT-PCR for influenza matrix (M) gene [17]. The positive swab pools were cultured in 10-day-old embryonated eggs. The allantoic fluid was collected 5 days post-inoculation, and tested for hemagglutination and viral RNA presence. Positive samples were forwarded to National Veterinary Services Laboratory (NVSL), Ames, Iowa for sub-typing using reference panel antisera for HA and neuraminidase antigen. The virus was characterized by NVSL to belong to the H2N2 subtype and was considered to be a low pathogenic strain by chicken pathogenicity testing. In addition, on the farm, the H2N2 infection of chickens was not found to be associated with an increase in morbidity or mortality.

Sequence analysis of the HA gene of influenza A/ck/PA/2004 virus

In order to determine the sequence of the HA gene of influenza A/ck/PA/2004 virus, we extracted RNA from allantoic fluid samples that were positive for HA activity and were RT-PCR positive by RNA easy mini kit (Qiagen Inc.). Full length cDNA was prepared by reverse transcription of extracted RNA followed by PCR amplification using the Superscript II One-Step Kit (Invitrogen 10928-034). Primers were designed to conserved 5′ and 3′ sequences in avian H2 HAs (5′H2 2004 SacI GAGAGAGCTCAGCAAAAGCAGGGGTTATACC and 3′H2 2004 XhoI GAGACTCGAGAGTAGAAACAAGGGTG). The resulting HA cDNA was cloned into the pCAGGS mammalian expression vector at the SacI/XhoI sites. Sequencing was carried out on an Applied Biosystems model 3700 automated sequencer at the Mount Sinai School of Medicine DNAcore facility. The HA gene of A/ck/PA/2004

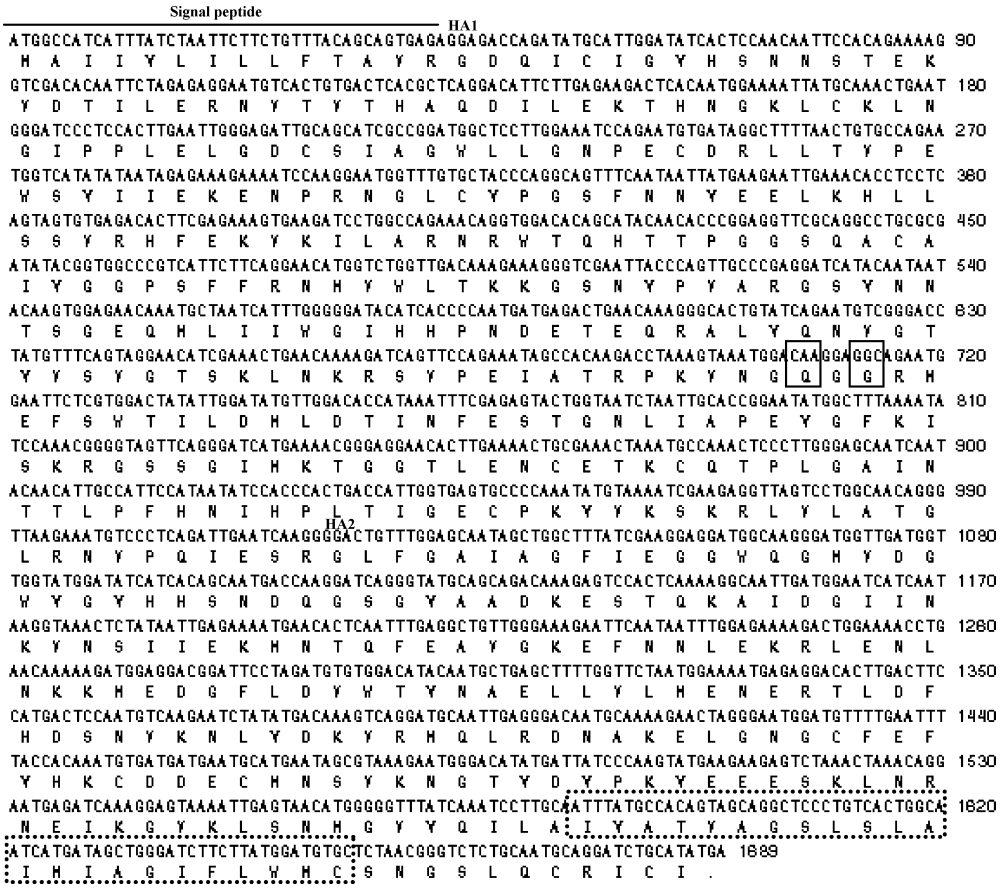

Fig. 1 Coding sequence and deduced amino acid sequence of the HA gene of A/ck/PA/2004. Dotted box encloses the predicted transmembrane domain. Solid boxes enclose amino acids 226 and 228 (H3 numbering) [33]

virus is 1,773 base pairs in length and codes for a 562 amino acid protein. Figure 1 shows the nucleotide sequence of the open reading frame with the predicted amino acid sequence above. The cleavage site in front of the HA2 domain was not consistent with those of H5 and H7 virus isolates that are highly pathogenic for gallinaceous poultry. These highly pathogenic viruses are characterized by multiple arginine residues in their HA cleavage sites. Cleavage is predicted to lead to the formation of a 325 amino acid HA1 domain and a 222 amino acid HA2 domain. Previous studies that analyzed sequences of human and avian H2 viruses determined that three amino acid positions near the receptor binding domain are conserved in all avian H2s but often mutated in the human adapted H2 viruses [3,4]. This suggests that these positions play a role in receptor specificity. As shown in Table 1 A/ck/PA/04 HA maintains the avian consensus at all three positions. However, since these studies mostly focused on H2s isolated from wild birds before 1980, it is of interest to study the receptor binding of a strain currently circulating in chickens.

Table 1 Critical amino acids for the receptor binding specificity of the influenza virus H2 and H3 HAs

	Amino acid number (H3 numbering)		
Virus isolate	138	226	228
A/ck/PA/2004 (avian H2)	A	Q	G
A/Japan/305+/57 (human H2)	A	L	S
A/duck/KH/273/78 (avian H2)	A	Q	G

A/ck/PA/04 HA preferentially binds α2,3 linked sialic acids

In order to investigate the receptor binding specificity of A/ck/PA/2004, we used the hemadsorption assay [18–20]. This assay exploits the fact that the sialic acid on the red blood cell surface can bind the influenza HA. Red blood cells from most sources contain a mixture of α2-3 and α2-6 linked sialic acid. In order to study differential binding to the these different forms of sialic acid, we first used a bacterial neuraminidase to remove all the sialic acid from chicken red blood cells and then used linkage specific sialyltransferases

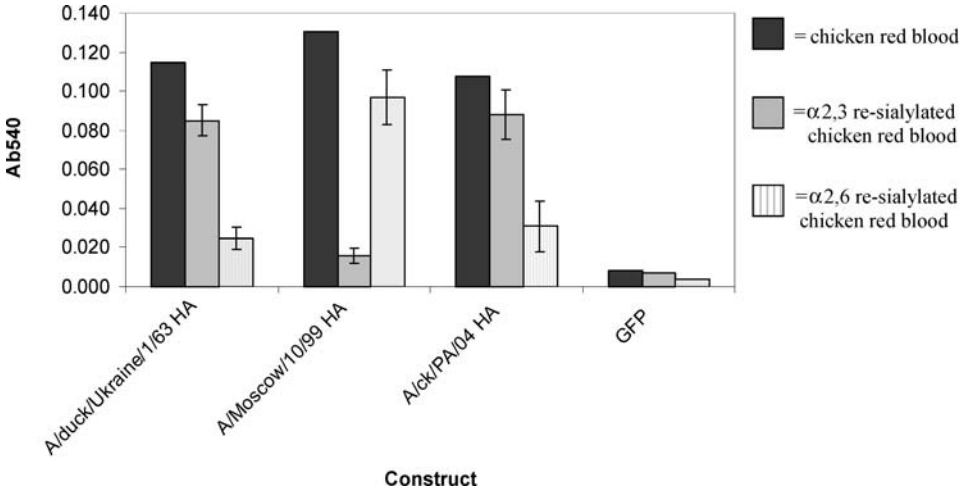

Fig. 2 A/ck/PA/2004 HA preferentially binds α2,3 sialic acid. Absorbance at 540 nm of untreated and re-sialylated CRBCs lysed after hemadsorption on 293T cells transfected with plasmids expressing A/ck/PA/2004 HA, A/duck/Ukraine/1/63 HA (avian influenza virus control), A/Moscow/10/99 HA (human influenza virus control) or GFP. Each bar represents the average of two independent experiments done in triplicate

to re-sialylate the red blood cells with exclusively α2-3 or α2-6 linked sialic acid [21]. Chicken red blood cells (CRBC) were obtained from CBT Farms (Chestertown, MD). Cells were washed twice in PBS and resuspended to 20% in 50 μl PBS. Sialic acid was removed from CRBCs by treatment with 50 mU *Vibrio cholerae* neuraminidase (VCNA) (Roche cat# 70008326), in the presence of 10 mM $CaCl_2$ at 37°C for 1 h. Cells were subsequently washed twice with PBS and resuspended in 50 μl PBS/1% BSA. For α2-3 re-sialylation, CRBCs were incubated with 1.5 mM CMP-sialic acid (Sigma cat# 8271) and 0.125 mU α2-3 sialyltransferase (Calbiochem cat# 566218) in a final volume of 50 μl for 30 min at 37°C. For α2-6 re-sialylation, CRBCs were incubated with 1.5 mM CMP-sialic acid and 0.480 mU α2-6 sialyltransferase (Calbiochem cat# 566222) in a final volume of 125 μl for 1 h at 37°C. Cells were washed twice with PBS and resuspended to 0.5% in 2 ml PBS.

Approximately 5.0×10^4 293T cells were transfected in suspension with 0.6 μg of pCAGGS-A/ck/PA/04-HA expression plasmid using Lipofectamine 2000 (Invitrogen cat# 11668019) according to the manufacturers instructions. As controls, we transfected cells with pCAGGS expression plasmids for the avian H3 HA from influenza A/duck/Ukraine/1/63 virus and for the human H3 HA of influenza A/Moscow/10/99 virus and for GFP. Cells were plated in 24 well poly-D-lysine coated plates with DMEM/10%FBS. After 6–12 h, fresh media containing antibiotics was added. Twenty-four h post-transfection cells were treated with 5 mU of VCNA in DMEM for 1 h at 37°C to remove sialic acid from the expressed HAs because previous studies showed that the sialic acid on the HA can interfere with the assay [22]. Cells were washed twice with PBS to remove excess VCNA and incubated with 200 μl of 0.5% enzymatically modified CRBCs for 30 min at 4°C. Cells were washed twice with DMEM to remove unbound red blood cells. Bound red blood cells were lysed in 150 μl 20 mM Tris-HCl pH 7.5, 177 mM NH_4Cl for 2 hours [20]. Absorbance of hemoglobin at 540 nm was measured using a plate reader in order to quantify the number of red blood cells bound.

Figure 2 shows that all three HA constructs bind well to unmodified CRBCs suggesting that all the HAs are well expressed on the cell surface. As expected the A/duck/Ukraine/1/63 HA binds best to the red blood cells with the avian influenza receptor, Siaα2-3Gal. On the other hand, the A/Moscow/10/99 HA has a preference for the human influenza receptor, Siaα2-6Gal. The HA from the newly isolated influenza A/ck/PA/2004 virus has a profile similar to the avian control virus and binds the avian influenza receptor, Siaα2-3Gal better than the human receptor, Siaα2-6Gal.

Phylogenetic analysis of the HA gene of influenza A/ck/PA/2004 virus

The HA gene sequence was aligned with 43 human, wild waterfowl, and shorebird isolate H2 HA genes by Clustal V (Lasergene, DNAStar, Madison WI). Phylogenetic analysis was performed with PAUP* 4.0b10 (Sinauer Associates, Inc., Sunderland, MA) using the maximum parsimony tree building method, with heuristic search with 100 bootstrap replicates. The phylogenetic tree is represented in Figure 3. A/ck/PA/2004 virus HA was closely related to HAs from viruses isolated from wild-birds in Europe and fell into the Eurasian clade of H2 HAs.

Discussion

Recovery from chicken of influenza A/H2N2 virus, a viral subtype that has been sporadically isolated from wild and domesticated ducks and was also a cause of the human

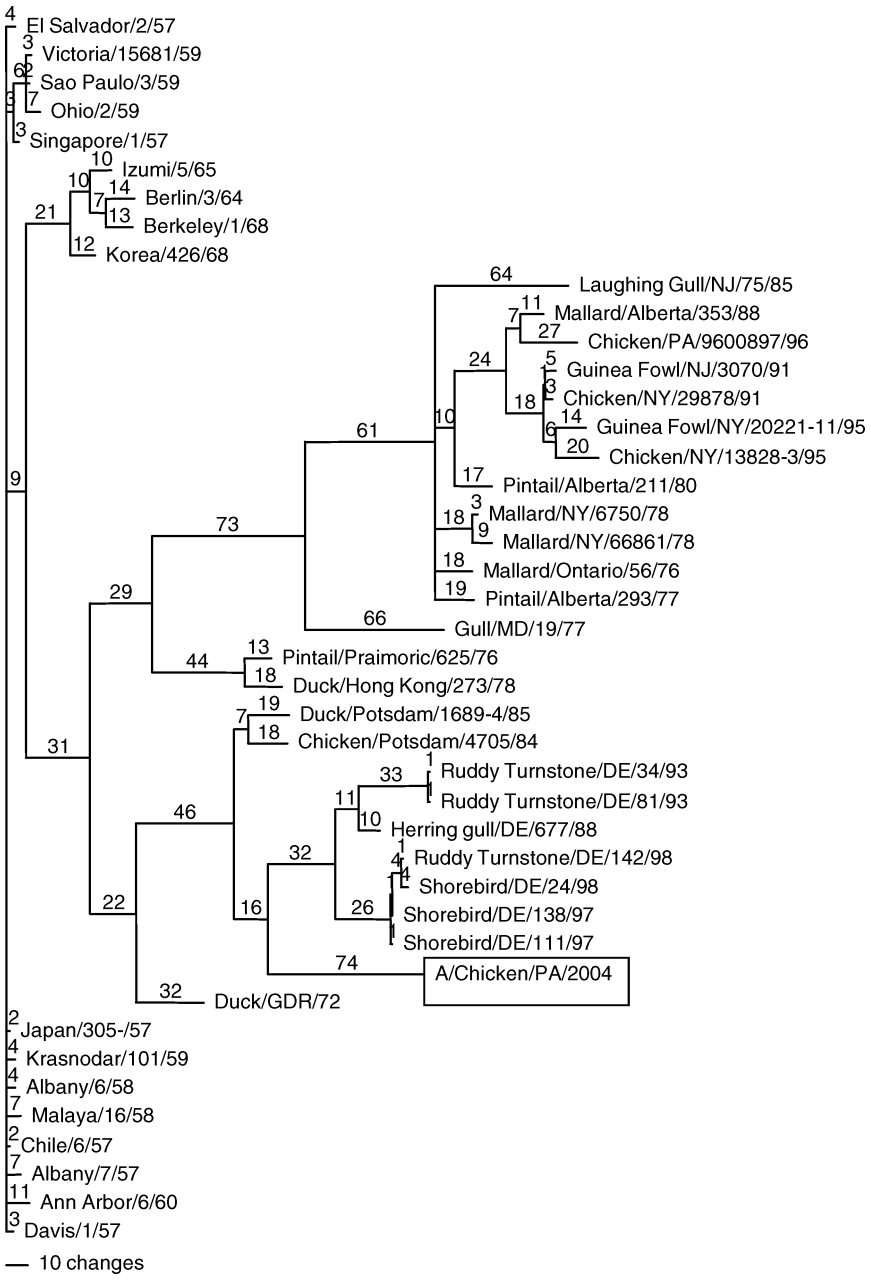

Fig. 3 Phylogenetic tree of selected human and avian H2 HA sequence. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) using maximum parsimony, heuristic search and 100 bootstrap replicates. A/ck/PA/2004 is boxed

pandemic in 1957 led us to test the receptor binding specificity of the recovered strain. The prevalence of this subtype in the United States between 1980–84 was less than 1% among the recovered bird isolates, which increased three fold in 1988, followed by a marked decline in later years [13,17]. In humans, this subtype has not been recovered since 1968. The phylogenetic analysis suggested that the recovered virus was most closely related to isolates that were isolated from wild waterfowl and shore birds but more distantly related to human isolates. Among H2 viruses described in gallinaceous birds, the recovered influenza A/ck/PA/2004 virus was most closely related to influenza A/ck/Potsdam/4705/84 virus (90.1% HA amino acid identity). Our studies indicate that the influenza A/ck/PA/2004 virus H2 falls into the Eurasian sublineage and demonstrates an avian (Siaα2-3Gal) influenza virus receptor binding specificity.

Current evidence suggests that differences in receptor binding specificities play a role in limiting infections in humans by avian viruses. A survey of the receptor binding specificity of 50 human and wild aquatic bird H2 and H3 HAs demonstrated that most human influenza viruses prefer Siaα2-6Gal while five human H2s from the study show a dual specificity for both Siaα2-6Gal and Siaα2-3Gal [3]. Sequence analysis of several of the isolates revealed that amino acid residues 226 and 228 play a critical role in determining the receptor-binding capabilities. HAs that bind Siaα2-6Gal

contain a leucine and serine at positions 226 and 228, respectively, whereas HAs that bind Siaα2-3Gal have glutamine and glycine at these positions. Further work on HAs of the H3 and H5 subtypes showed that the receptor binding specificity could be switched solely by changing these amino acids [23–25]. X-ray crystallography studies of H3 HAs partially explain the reason that a few amino acids can switch receptor binding specificity. The structures show that the presence of a glutamine at position 226 in the receptor binding pocket narrows the geometry of the pocket and favors the orientation of the α2-3 linked sialic acid [26,27]. The HA from A/ck/PA/2004 contains 226Gln and 228Gly, and, as predicted from the studies of earlier H2s, it has specificity for Siaα2-3Gal. It has been shown that chicken influenza viruses do not have precisely the same requirements for Siaα2-3Gal binding as other avian influenza viruses. In particular, sulfation and other moieties on the carbohydrate chain play a role [28, 29]. Further studies of the receptor specificity from A/ck/PA/2004 would be needed to know if it recognizes mainly wild bird or gallinaceous bird receptors. However, the sequence of the NS gene from A/ck/PA/2004 is consistent with a recent introduction of this virus into chickens from ducks (unpublished results).

The importance of receptor binding specificity in infection has been substantiated in animal models. In experiments with ferrets, which like humans have a predominance of Siaα2-6Gal on their tracheas, a human virus with an HA with a single amino acid change (L226Q) that increases its affinity for Siaα2-3Gal binding and decreases its Siaα2-6Gal binding is attenuated [30,31]. Sequence analysis, structural, and animal model studies are consistent with the hypothesis that before a human pandemic virus strain can arise the HA of the new virus must acquire the ability to bind to the human sialic acid receptor. However recent infections of humans directly with avian influenza viruses question the absolute need for Siaα2-6Gal specificity for human infection. In 1997 and recently, humans have been infected by highly pathogenic avian influenzas of the H5N1 subtype causing severe illness and death. In the case of the 1997 outbreak, HAs of viruses isolated from human cases maintained the avian Siaα2-3Gal receptor binding specificity [32]. Although humans were presumably infected directly from contact with birds, there was no evidence of human to human viral transmission, a prerequisite for developing a new pandemic. Thus, the ability to bind to Siaα2-6Gal may be needed for viral propagation in human populations. Our paper and others demonstrates that H2N2 viruses similar to the one that caused the 1957 pandemic continue to circulate in the wild and in domestic poultry [13–15]. Since influenza virus accumulates numerous mutations during replication and receptor binding specificity can change following minimal amino acid changes in the receptor binding pocket it is conceivable that an HA from an avian influenza virus could gain the ability to bind to the human influenza virus receptor. In light of this fact, it is important to survey current avian influenza viruses for their ability to bind human Siaα2-6Gal which could increase their potential as human pathogens.

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