Virology 412 (2011) 401–410

Contents lists available at ScienceDirect

Virology

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Receptor specificity of subtype H1 influenza A viruses isolated from swine and humans in the United States

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ARTICLE INFO

Article history:
Received 30 November 2010
Returned to author for revision
21 December 2010
Accepted 13 January 2011
Available online 18 February 2011

Keywords:
Influenza
Swine
Human
H1 hemagglutinin
Receptor
Binding
Sialic acid

ABSTRACT

The evolution of classical swine influenza viruses receptor specificity preceding the emergence of the 2009 H1N1 pandemic virus was analyzed in glycan microarrays. Classical swine influenza viruses from the α, β, and γ antigenic clusters isolated between 1945 and 2009 revealed a binding profile very similar to that of 2009 pandemic H1N1 viruses, with selectivity for α2-6-linked sialosides and very limited binding to α2-3 sialosides. Despite considerable genetic divergence, the 'human-like' H1N1 viruses circulating in swine retained strong binding preference for α2-6 sialylated glycans. Interspecies transmission of H1N1 influenza viruses from swine to humans or from humans to swine has not driven selection of viruses with distinct novel receptor binding specificities. Classical swine and human seasonal H1N1 influenza viruses have conserved specificity for similar α2-6-sialoside receptors in spite of long term circulation in separate hosts, suggesting that humans and swine impose analogous selection pressures on the evolution of receptor binding function.
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Introduction

A novel influenza A virus subtype H1N1, discovered in April 2009, spread globally in the human population causing a pandemic that officially was declared by the World Health Organization on June 11, 2009. Phylogenetic analysis of the 2009 pandemic H1N1 virus indicated that the genome was a combination of gene segments that had not been identified previously in animal or human influenza viruses (Garten et al., 2009; Smith et al., 2009). The HA gene of the pandemic H1N1 belongs to the classical swine lineage whose ancestry has been traced to viruses similar to the human pandemic of 1918–19 (Garten et al., 2009). This lineage is known as classical swine influenza to distinguish it from other viruses of human or avian origin that were introduced into pigs more recently, giving rise to 'human-like' and 'avian-like' swine influenza lineages (Brown, 2000; Brown et al., 1997; Guan et al., 1996; Karasin et al., 2006, 2002, 2004; Pensaert et al., 1981; Vincent et al., 2006, 2009, 2008; Webby et al., 2004; Yu et al., 2009). The classical swine H1N1 virus has continued to circulate in pigs without interruption since 1930 (Brown, 2000; Vincent et al., 2008). Around 1998 this virus reassorted with human and avian viruses in pigs to give rise to the 'triple reassortant' (H3N2) swine influenza viruses (Karasin et al., 2000; Webby et al., 2000; Zhou et al., 1999). Subsequent reassortment with a circulating classical swine influenza virus resulted in the transfer of the classical H1 HA (with the N1 NA or alone) to yield H1N1 and H1N2 triple reassortant swine viruses (Karasin et al., 2002; Vincent et al., 2006). The HA gene of these swine viruses belongs to the classical swine lineage, and has evolved into three distinct phylogenetic and antigenic clusters, termed α, β and γ, which have co-circulated in the United States since 2000 (Vincent et al., 2006). A triple reassortant swine virus with the classical swine HA (γ cluster) eventually acquired two genes (M and NA) from the Eurasian 'avian-like' swine viruses to give rise to the 2009 pandemic H1N1 virus (Garten et al., 2009).

Human infections with classical swine H1N1 viruses have been documented since the early 1970s (Dowdle, 1997; Gaydos et al., 1977; Myers et al., 2007, 2006). These infections were detected very infrequently; up to one or two cases were reported each year in the United States, without sustained transmission in the community. Increasingly frequent human infections with 'triple-reassortant' swine

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2011.01.015

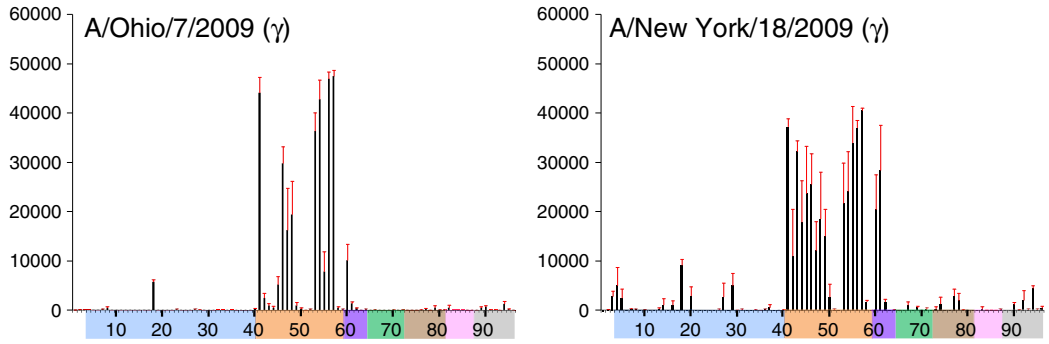


Fig. 1. Receptor binding specificity of 2009 H1N1 pandemic influenza viruses. Glycan microarray analysis of 2009 H1N1 pandemic viruses, as listed in Table 1. The identity of each numbered glycan (abscissa) is provided in Supplementary Table S1. Different categories of glycans are color-coded as follows: No color, sialic acid; blue, α2-3 sialosides; orange, α2-6 sialosides, violet, mixed α2-3/α2-6 biantennaries; green, N-glycolylneuraminic acid-containing glycans; brown, α2-8 linked sialosides; pink, β2-6 linked as well as 9-O-acetylated sialic acids; grey, asialo glycans. The vertical bars denote fluorescent binding signal intensity (ordinate) and error T bars indicate the standard error. Glycan #18 is a ligand for the primary sheep antibody; therefore this fluorescence signal is regarded as inconclusive (Supplementary Figure 1). Greek letter in parentheses indicates the antigenic cluster classification reported by Vincent et al. (Lorusso et al., 2010; Vincent et al., 2009).

influenza A (H1) have been noted since 2005 in the United States (Newman et al., 2008; Shinde et al., 2009). The determinants of the rising number of human infections remain unclear, although improved virologic surveillance at the human-animal interface, expanded subtype-level testing capacity at public health laboratories and new reporting requirements for novel influenza A could have played a role (Shinde et al., 2009).

Table 1
Influenza viruses analyzed in this study.

Virus lineage and name	HA sequence	Cluster	Host	Passage
Classical swine lineage				
Pandemic isolates:				
A/Ohio/07/2009		γ	Human	C3*
A/New York/18/2009	222 D	γ	Human	C2
A/New York/18/2009 (rg variant)	222 G	γ	Human	E2
A/New York/18/2009 (rg variant)	223 R	γ	Human	E2
A/New York/18/2009 (rg variant)	127 E	γ	Human	E2
A/Texas/05/2009	155 G	γ	Human	C6
A/Texas/05/2009 (variant)	155 E	γ	Human	C6
A/Mexico/Indre/4114/2009	222 G	γ	Human	E5
A/New York/04/2009	222 G	γ	Human	E4
A/Michigan/10/2009	222 N	γ	Human	C2
Recent swine isolates				
A/Iowa/02/2009		γ	Human	C2
A/Ohio/01/2007		γ	Human	C4
A/Ohio/02/2007		γ	Human	C5
A/Illinois/33295/2007		γ	Human	C5
A/swine/Ohio/02026/2008		γ	Swine	C3
A/swine/Missouri/02060/2008		γ	Swine	C3
A/swine/Iowa/02096/2008		β	Swine	C3
A/swine/North Carolina/02023/2008		γ	Swine	C3
A/Minnesota/03/2008	222 N	β	Human	Rx/C2
A/Texas/14/2008	222 N	β	Human	M1/C2
A/South Dakota/03/2008		β	Human	M2/C4
A/Missouri/04/2006		β	Human	M2/C5
A/swine/Nebraska/02013/2008		β	Swine	C4
A/swine/North Carolina/02084/2008		β	Swine	C3
A/swine/Minnesota/02093/2008		α	Swine	C3
A/swine/Minnesota/02053/2008		α	Swine	C3
Early swine isolates				
A/New Jersey/8/1976	222 G	α	Human	E8
A/swine/Wisconsin/1/1968	222 G	α	Swine	X/C1
A/Swine/Iowa/1945	222 G	α	Swine	X/C1
'Human-like' H1				
A/swine/Iowa/02039/2008	187 N		Swine	C3
A/swine/Minnesota/02011/2008			Swine	C3
Human seasonal				
A/Brisbane/59/2007 (rg)	187 D		Human	S2
A/Brisbane/59/2007 (rg variant)	187 N			Cx/C7
A/New Caledonia/20/1999 (rg)	187 D		Human	Lon2
A/New Caledonia/20/1999 (rg variant)	187 N			E2
A/New Caledonia/20/1999 (rg variant)	187 V			E2

*Key: C = MDCK ATCC, Lon = MDCK London, M = primary monkey kidney, S = MDCK-SIAT1, R = R-Mix, E = egg, X = unknown.

Interspecies transmission in the opposite direction, from humans to swine, also has been documented. The HA of H1N1 human seasonal influenza has been identified in reassortant swine isolates since 2003 (Karasin et al., 2006). This virus lineage, termed 'human-like' swine H1N1 or H1N2 viruses, is antigenically distinct (comprising the δ cluster) from classical swine viruses. These viruses have also circulated in the United States in recent years (Vincent et al., 2009) and provide an opportunity to study the seasonal human HA after cross-species transmission from man to swine.

The influenza pandemics of 1957 and 1968 were caused by viruses with HA genes of avian origin (Schäfer et al., 1993; Scholtissek et al., 1978; Webster et al., 1997). These avian HAs have a strong binding preference for glycan receptors with sialic acids in α2-3 linkage to galactose. Mutations in the receptor binding site of the HA were required for a functional switch towards recognition of α2-6 sialoside receptors, which is thought to be critical for virus transmissibility among humans (Connor et al., 1994; Rogers and Paulson, 1983; Rogers et al., 1983; Stevens et al., 2006; Xu et al., 2010b). Interestingly, a recent study with classical swine influenza viruses isolated up to 2003, revealed an α2-6 sialoglycan receptor preference as typically seen for human influenza viruses (Gambaryan et al., 2005), suggesting that major changes in receptor specificity would not be required for infection and transmission in humans. Genetic and antigenic analyses have suggested the HA of 2009 pandemic H1N1 viruses to be related most closely to that of the γ cluster swine viruses (Vincent et al., 2010). However, the only comparison reported to date analyzed the receptor binding specificity of a β cluster 2006 H1N1 classical swine virus isolated from a human infection (Childs et al., 2009).

In this study, we have performed a comprehensive analysis of the glycan binding profiles of multiple isolates from each of the three classical swine influenza clusters to determine the evolution of receptor specificity among these and 2009 H1N1 pandemic viruses. These studies were also complemented by analyzing the receptor specificity of both swine viruses that had transmitted to humans, and conversely 'human-like' H1N1 viruses isolated from swine. Additional studies were performed to shed light on the impact of amino acid changes close to the receptor binding site in H1N1 viruses isolated in egg or cell cultures, essential for the integration of these results. We found a remarkable similarity between the receptor specificity of classical swine isolates and pandemic 2009 H1N1, suggesting that receptor specificity alone has not restricted the transmissibility of classical swine H1N1 viruses among humans.

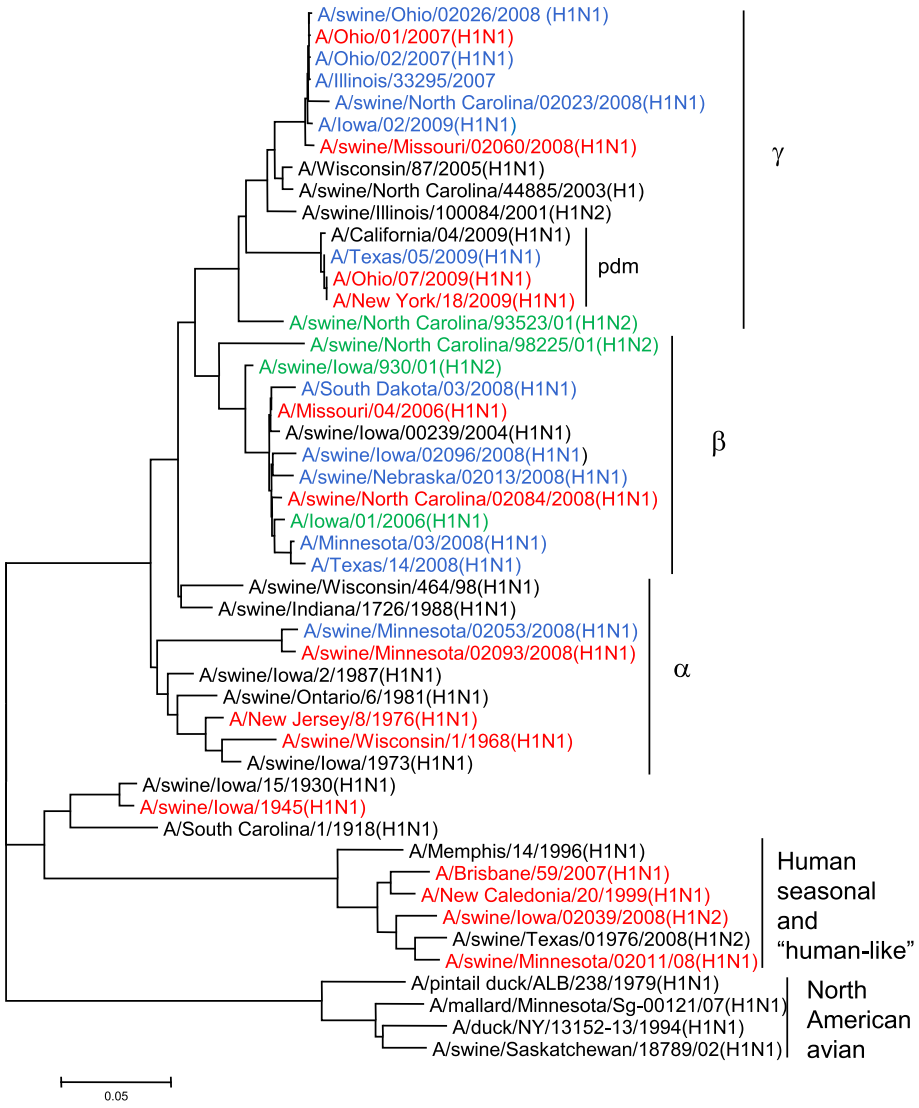


Fig. 2. Phylogenetic analysis of H1 HA genes from swine and 2009 pandemic H1N1 viruses. Phylogenetic analysis of selected H1 HA genes descending from the HA of 1918 pandemic viruses. The phylogenetic tree was constructed by Neighbor-Joining using MEGA software (Kumar et al., 2004) and includes an outgroup of North American avian H1 genes. Viruses highlighted in red font were analyzed in the glycan array and shown in Figs. 1, 3, 4 and 5), whereas viruses in blue font were analyzed in the array and shown in the Supplementary Figures. Viruses analyzed in previous studies are indicated in green fonts (Childs et al., 2009; Gambaryan et al., 2005). Greek letter in parentheses indicates the antigenic cluster classification reported by Vincent et al. (Lorusso et al., 2010; Vincent et al., 2009).

Results

Receptor binding characteristics of 2009 pandemic H1N1 influenza viruses

Microarrays displaying a library of sialylated glycans attached to a silica surface mimic viral receptors on cell surfaces and are a powerful tool to study the receptor binding repertoire of influenza viruses (Blixt et al., 2004; Stevens et al., 2008). We used this approach to analyze 2009 pandemic H1N1 viruses from early cases in the United States, which were isolated and propagated in MDCK cells. The A/Ohio/07/2009 virus revealed a pattern of highly specific binding to α2-6-linked sialyllactosamine (Fig. 1, glycan #53, 54), a longer α2-6 sialylated di-N-acetyllactosamine (glycan #56), as well as related α2-6-linked bi-antennary glycans (#46, 47, 48); all of which were among the N-glycan structures of a cultured human bronchial epithelial cell line (Chandrasekaran et al., 2008). The A/Ohio/07/2009 virus also bound to an α2-6 sialylated tri-N-acetyllactosamine glycan in which the two proximal (reducing end) lactosamines are 1-3 fucosylated (#57) as well as a sulfated α2-6-linked sialyllactosamine (#41) and less strongly to α2-6 sialylated N, N'- diacetyllactosediamine (LacDiNac) (#55); glycans previously detected in human tissues (Crottet et al., 1996; Degroote et al., 2003). Interestingly, another MDCK-propagated 2009 pandemic H1N1 virus isolated from a different outbreak; A/New York/18/2009, bound to all the glycans recognized by the A/Ohio/07/2009 isolate as well as closely related structures, including branched α2-6 glycans (Fig. 1, glycans #42–45) and α2-3/6 hybrid bi-antennary sialosides (#60–61). Very limited binding to α2-3-linked sialosides was detected in either virus. The binding signal detected in glycan #18 should be interpreted with caution due to non-specific results with primary and secondary control antibodies used for detection (Supplementary Fig. 1).

Receptor specificity of classical H1N1 swine influenza viruses

To determine whether the receptor specificity of the pandemic H1 HA was altered in concert with transmission to the human population, we analyzed a panel of North American swine influenza viruses bearing the classical swine HA, isolated during 2006–2009, as well as representative older viruses, all from the α, β and γ clusters (Table 1, Fig. 2). The virus panel included isolates from swine and sporadic

infections in people (Shinde et al., 2009; Vincent et al., 2010). Viruses isolated and propagated in MDCK cells were used herein, except for swine viruses isolated in or before 1976, from which only egg isolates were available.

In general, swine viruses with classical HA from the α, β, and γ clusters and isolated between 2006 and 2009 showed binding profiles resembling 2009 pandemic H1N1 viruses; i.e. highly specific for α2-6-linked sialosides (Fig. 3A) and very limited affinity to α2-3 sialosides. These viruses recognized short and long α2-6-linked sialyllactosamine chains (Fig. 3A and Supplementary Fig. 2, glycans #53, 54, 56, and 57). Sulfated α2-6 sialyllactosamine and α2-6 sialyl LacDiNAc (glycans #41 and 55) were also preferred ligands for virtually all classical H1N1 swine viruses. All of the viruses showed good to moderate binding to α2-6-sialylated bi-antennary glycans (glycans #46, 47, and 48), whereas binding to the α2-6-sialylated branched glycans (glycans #43, 44, and 45) was more variable in the isolates tested. Binding to α2-3/2-6 bi-antennary sialosides (glycans #60–61) was detected in most of the isolates. The binding profiles of β and γ cluster viruses isolated from swine were indistinguishable from those of human isolates from the same cluster.

Classical swine influenza viruses belonging to the α cluster and isolated in or before 1976 in eggs (A/swine/Wisconsin/1/1968 and A/New Jersey/8/1976) bound to the set of α2-6-linked sialylated glycans recognized by contemporary swine viruses, but in addition, they bound α2-3 linked sialosides, including 6′-sulfated and non-sulfated sialyl Lewis X (Fig. 3B, glycans # 4–8, 30) and α2-3-linked di-sialosides (glycans #9–10). A similar specificity pattern for binding α2-3 and α2-6 glycans; i.e. dual specificity, also is found in the ancestor virus, A/swine/Iowa/1945. However, an α cluster virus isolated in cell culture (A/swine/Minnesota/02093/2008 in Fig. 3A) lacked binding to these additional α2-3-linked glycans suggesting the possible role of isolation and propagation hosts in selection of receptor variants, and in agreement with recent studies showing that isolation of classical swine influenza viruses in eggs selects for variants with increased binding to 2-3 sialoglycans (Gambaryan et al., 2005).

The similarity between the glycan binding patterns of human and swine viruses isolated in MDCK cells suggests that no further selection of receptor specificity was necessary for a swine virus with α2-6 binding preference to be transmitted to a human host. Interestingly, extensive divergence of swine H1N1 viruses into α, β and γ clusters involving many HA amino acid substitutions (Supplementary Table S2) which altered their antigenic properties (Vincent et al., 2010, 2006) have resulted in only minimal changes in receptor binding properties.

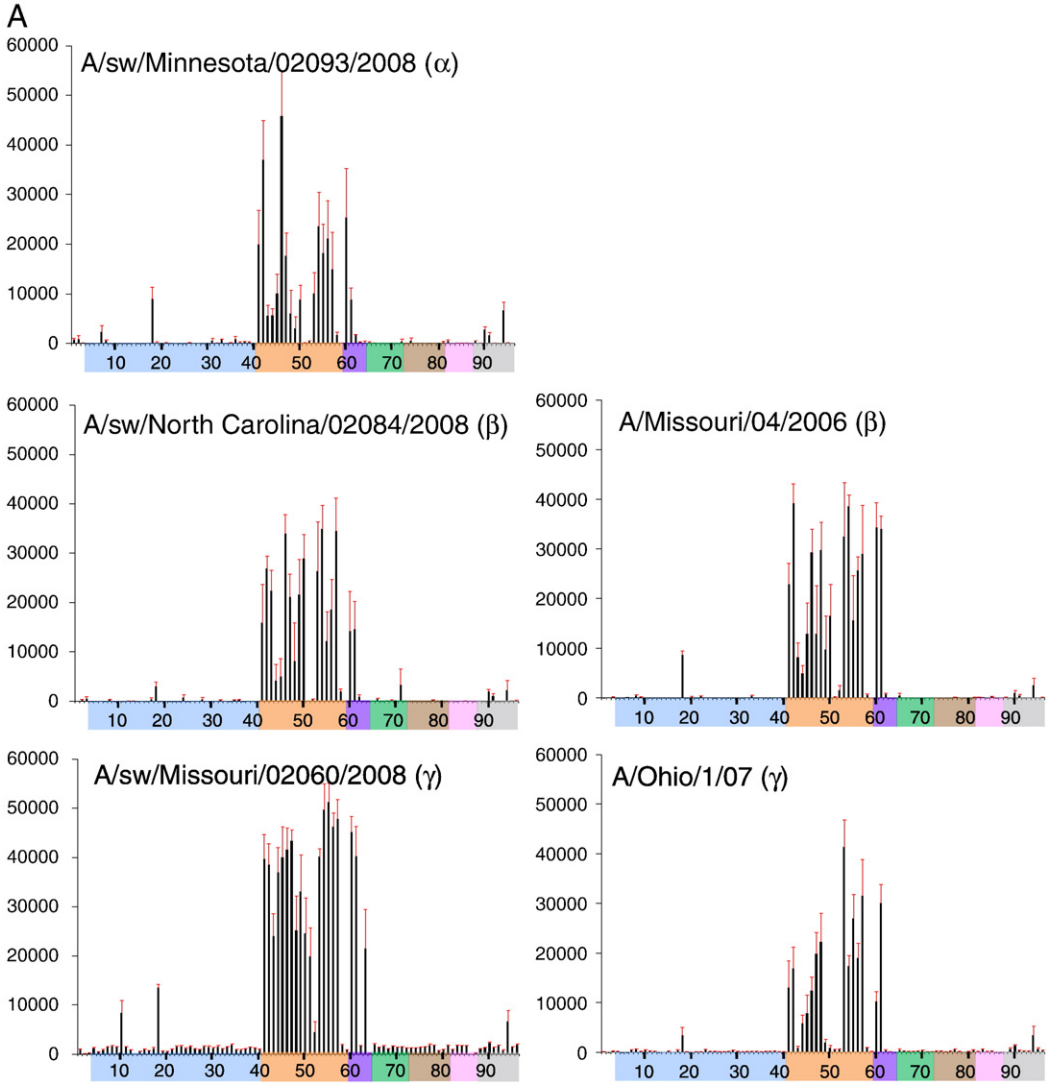


Fig. 3. Receptor binding specificity of swine H1N1 viruses. Glycan microarray analysis of (A) recently and (B) older swine H1 viruses, as listed in Table 1. Other details of the glycan array were described in Fig 1.

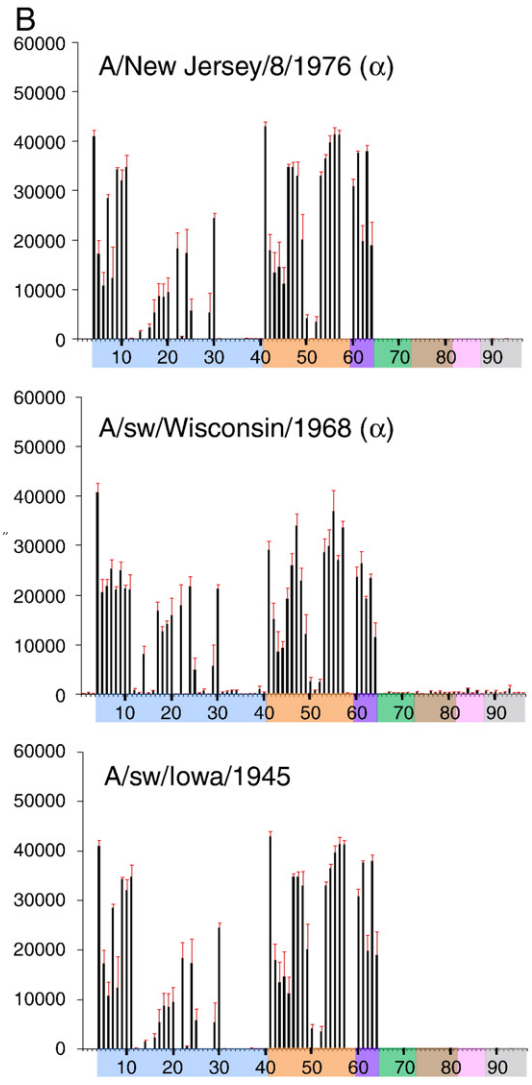


Fig. 3 (*continued*).

Receptor binding characteristics of 'human-like' viruses isolated from swine

The similar sialoside binding profiles of classical swine H1N1 viruses and 2009 pandemic H1N1 viruses prompted us to analyze the receptor profiles of viruses transmitted in the reverse direction, i.e., the so-called 'human-like' H1N1 and H1N2 swine influenza viruses currently circulating in North American herds (Karasin et al., 2006; Vincent et al., 2009). To determine the impact of human-to-swine transmission on the evolution of receptor specificity we first analyzed the human H1N1 viruses that carried the precursor HAs. The human A/New Caledonia/20/1999 virus, a close ancestor of 'human-like' swine viruses, recognized a similar set of α2-6-linked sialoglycans, to those bound by swine influenza viruses with classical swine HA and the 2009 pandemic H1N1 virus (Fig. 4).

Analysis of the 'human-like' H1N1 viruses isolated from swine indicated that they retained their strong binding preference for α2-6 sialylated glycans after transmission and sustained circulation in swine; these viruses bound to the set of α2-6 linked sialosides recognized by human seasonal H1N1 virus A/New Caledonia/20/1999. In addition, these viruses showed slightly increased binding to sialyl Lewis X structures (Fig. 4, glycans #30–33), which are present in the human respiratory tract (Allahverdian et al., 2006; Groux-Degroote et al., 2008). One of the viruses, A/swine/Iowa/02039/2008 revealed additional binding to sulfated α2-3 linked sialosides (including sulfated sialyl Lewis X) (Fig. 4, glycans #4–7), and a long α2-3 linked sialoside (#24). However, the HA of this isolate has a D187N substitution, previously reported to affect receptor binding specificity (Gambaryan et al., 1999) (see next section). These findings suggest that the receptor repertoire of the HA from recent 'human-like' swine viruses resembles those of human seasonal H1N1 viruses and classical swine H1N1 viruses with an incipient binding to α2-3 linked sialosides.

Receptor binding specificity of H1 subtype viruses with acquired mutations at antigenic or receptor-binding sites

Numerous studies have documented that the host cells supporting influenza replication *in vivo* or *in vitro* can select variant viruses with amino acid substitutions near the receptor binding site of the HA (Couceiro et al., 1993; Ito et al., 1997; Pyhala et al., 1988; Rogers et al., 1985; Vines et al., 1998). For example, human H3N2 clinical specimens will yield different sequence variants when propagated in MDCK cells or in embryonated eggs (Stevens et al., 2010). Similarly, classical swine H1N1 viruses isolated in eggs have changes at positions 187 and/or 222 (using H1 HA numbering of the mature protein; equivalent to 190 and 225 in H3 numbering or crystallographic annotation systems) which modulate their receptor specificity (Gambaryan et al., 2005; Takemae et al., 2010). To understand the potential significance of these mutations on receptor binding, we evaluated the receptor specificity of a representative set of variant viruses with HA mutations at putative host-selected sites (Table 1).

The most frequent changes in the vicinity of the receptor binding pocket of the HA of egg-grown 2009 pandemic H1N1 viruses include D222G, Q223R and D127E (Robertson et al., 2011). Egg-grown 2009 pandemic H1N1 viruses A/Mexico/Indre/4114/2009, and A/New York/04/2009 with a D222G substitution revealed dual α2-3 and α2-6 binding specificity (Supplementary Fig. 3). Binding to α2-3-linked sialosides was substantially increased, whereas binding to α2-6-linked glycans remained unchanged relative to A/Ohio/07/2009 and A/New York/18/2009 cell-grown viruses (Fig. 1). To further investigate whether increased α2-3 sialoside binding was a direct consequence of the D222G mutation, we analyzed a reverse genetics (rg) virus pair derived from A/New York/18/2009 (wildtype and D222G mutant). As shown in Fig. 5A, the A/New York/18/2009-D222G rg virus revealed a minor decrease in binding to a few α2-6 branched sialosides (Fig. 5A, glycans #42–44), paralleled by increased binding to multiple α2-3-linked glycans, including di-sialoside (glycans #9–10), bi-antennary (glycan #11), long chain α2-3 sialyllactosamine (glycans # 22, 24, and 25) and sialyl Lewis X (glycans #31–33), as compared to the parental A/New York/18/2009 virus. On the other hand, the rg derived virus A/New York/18/2009-Q223R, another frequent mutation detected in egg isolates, showed an inverted sialic linkage specificity: complete loss of binding to α2-6-linked siaolosides coinciding with newly acquired binding activity for a broad range of α2-3-linked sialylglycans (Fig. 5A). In contrast to the significant binding changes noted with D222G and Q223R, the D127E substitution showed minimal change in binding profiles as compared to the original human virus (Fig. 5A).

Although mutations at position 223 in the HA of pandemic 2009 H1N1 viruses were only associated with egg adaptation, mutations at position 222 (D222G and D222N) were also identified in many clinical specimens collected during the 2009 pandemic and with higher frequency among samples from severe and fatal cases (Chutinimitkul et al., 2010; WHO, 2010). A human 2009 pandemic H1N1 virus isolate with D222N in HA, A/Michigan/10/2009, revealed a very slight increase in binding to α2-3-linked sialosides (Supplementary Fig. 4). It will be important to investigate whether D222G and D222N mutations and the concomitant binding of α2-3 sialosides might contribute to increased influenza severity in humans.

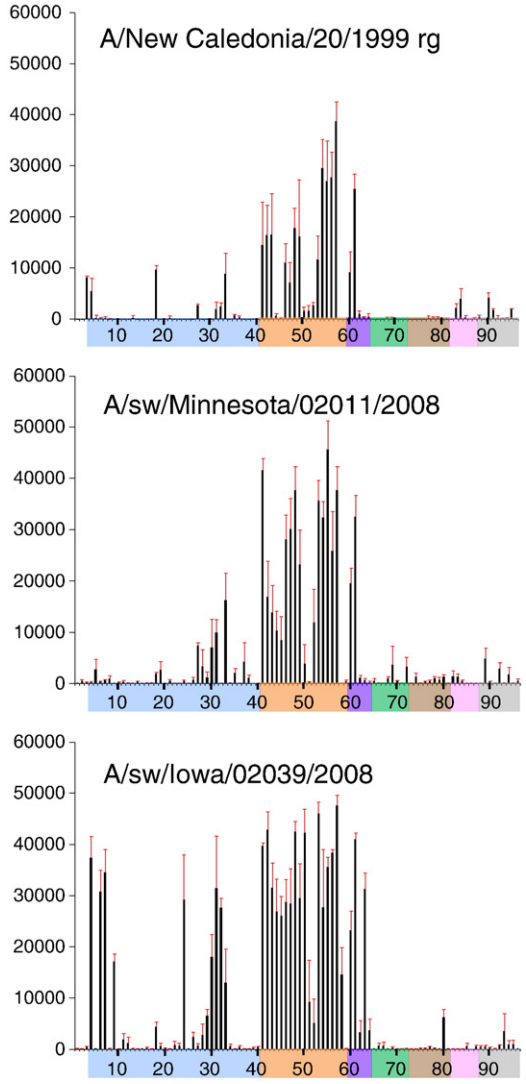


Fig. 4. Receptor binding specificity of 'human-like' H1N1 isolates. Glycan microarray analysis of 'human-like' H1N1 viruses, as listed in Table 1. Other details of the glycan array were described in Fig. 1.

In contrast to the frequent structural changes at positions 222, 223, and 127 among swine and pandemic H1 HAs, the seasonal H1 HA often acquired substitutions at position 187 (D187N or D187V) (Gambaryan et al., 1999). The impact of these mutations on receptor specificity was investigated by analyzing rg-derived A/New Caledonia/20/1999 viruses with D187N and D187V in HA. Both viruses retained binding to the α2-6-linked sialosides recognized by the wildtype viruses, but in addition they bound to a variety of α2-3-linked sialosides, including fucosylated (Fig. 5B, glycans # 28–33) and sulfated 2-3-linked sialosides, as well as sialyl Lewis X (#4–7). A more recent human seasonal isolate, A/Brisbane/59/2007, also showed a similar dual specificity binding pattern when the D187N substitution was present in the HA (Fig. 5B), confirming previous findings regarding the critical role of position 187 in the specificity of subtype H1N1 influenza viruses for different sialosides (Gambaryan et al., 1999; Matrosovich et al., 2000; Stevens et al., 2006; Takemae et al., 2010).

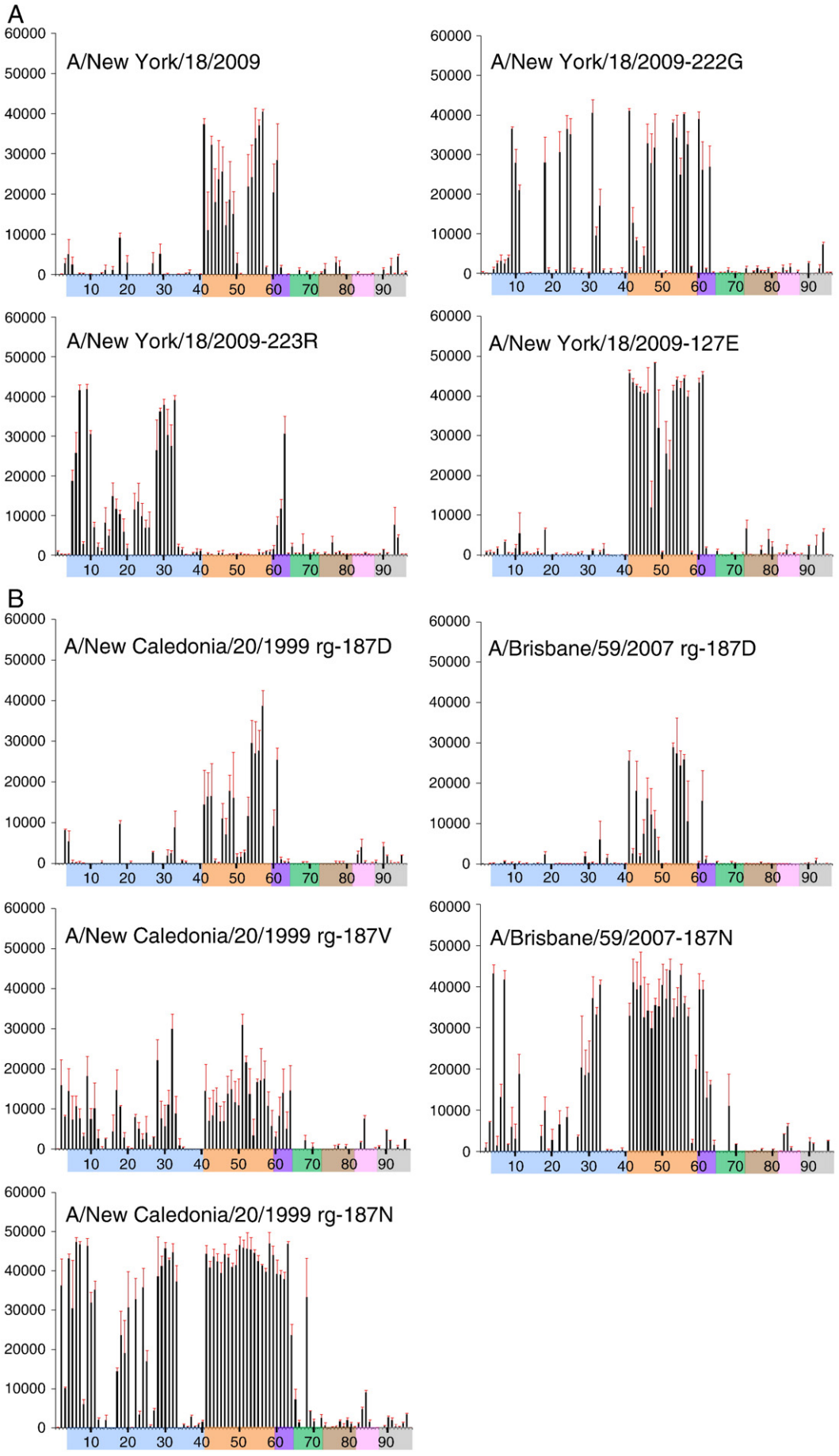
In addition to changes in the 220 loop, host-driven selection of variant 2009 pandemic H1N1 viruses often yielded mutations at the top of the globular head of the HA which were reported to modulate interactions with host cell membranes (Gambaryan et al., 1998; Xu et al., 2010a; Yang et al., 2010). Several mutations in the region have been reported so far, including K153E, K154E, G155E, N156D/S/K/T (Liu et al., 2010). Analysis of the G155E variant of A/Texas/05/2009 H1N1 pandemic virus revealed only a slightly increased binding affinity for the same glycans bound by the parental virus (Supplementary Fig. 5). Interestingly, amino acids 153–156 are located in antigenic site Sa and mutations at these positions resulted in greatly altered reactivity in HI tests with ferret antisera (Kilbourne et al., 1988). Taken together, these results further emphasize the role of the laboratory host used for isolation and propagation of swine and human influenza viruses in selecting variants with altered receptor binding characteristics that could affect the interpretation of receptor binding studies.

Discussion

Although the 2009 pandemic H1N1 virus contained a new combination of gene segments descending from Eurasian and North American triple reassortant viruses previously circulating in swine, the factors that contributed to the generation of the pandemic virus remain unclear. The animal hosts that supported the replication of influenza viruses immediately preceding their pandemic emergence in 1918, 1957 or 1968 were never directly identified. However, the detection of genes of avian and human influenza origin in the previous pandemic viruses led to the hypothesis that pigs served as a 'mixing vessel' for the generation of human-avian reassortant viruses (Laver and Webster, 1979; Scholtissek et al., 1985). This hypothesis is further supported by the detection of both α2-3 and α2-6-linked sialosides in the respiratory tract of swine, which would allow co-infections with avian and human viruses (Ito et al., 1998). The precursor HAs of the 1957 and 1968 pandemic viruses switched their receptor specificity to establish sustained transmission in the new host (Rogers and Paulson, 1983; Xu et al., 2010b). However, the role of receptor binding specificity in the emergence of 2009 pandemic H1N1 lacks detailed characterization. A study by Maines et al. indicated that pandemic H1N1 viruses bind specifically to α2-6 sialoside receptors, but noted a weak binding affinity that was correlated with suboptimal droplet transmission in ferrets (Maines et al., 2009). In contrast, recent microarray studies indicated that the receptor binding repertoire of pandemic H1N1 viruses is distinct from that of seasonal H1N1; besides strong binding to α2-6 sialosides, pandemic viruses displayed substantial interaction with α2-3 sialoside receptors, with overall similarity to the profiles of classical swine H1N1 viruses isolated in eggs (Childs et al., 2009; Liu et al., 2010). In summary, a consensus regarding the potential role of receptor specificity in the transmission of swine viruses to humans resulting in sporadic infections or in the emergence of 2009 pandemic H1N1 is lacking. Furthermore, there has not been adequate information on the receptor binding specificity of human H1N1 viruses that have transmitted to pigs and established the so-called 'human-like' H1N1 lineage after reassorting with triple-reassortant swine viruses to form the antigenically and genetically distinct δ cluster (Karasin et al., 2006; Lorusso et al., 2010; Vincent et al., 2009).

In this study, we analyzed the receptor binding properties of recent H1N1 or H1N2 swine and human (seasonal and pandemic) viruses that switched hosts in both directions and established sustained chains of transmission in the new host. We selected human and swine viruses from the United States, comprising α, β, and γ clusters, which were isolated and propagated exclusively in MDCK. We found that swine viruses with the classical swine HA from three different evolutionary clusters bound predominantly to a set of

Fig. 5. Receptor binding specificity of H1 subtype viruses with mutations near the receptor binding site. Glycan microarray analysis of (A) 2009 pandemic and (B) human seasonal H1N1 virus varients with substitutions near the receptor binding site, as listed in Table 1. Other details of the glycan array were described in Fig. 1.


A
A/New York/18/2009
A/New York/18/2009-222G
A/New York/18/2009-223R
A/New York/18/2009-127E
B
A/New Caledonia/20/1999 rg-187D
A/Brisbane/59/2007 rg-187D
A/New Caledonia/20/1999 rg-187V
A/Brisbane/59/2007-187N
A/New Caledonia/20/1999 rg-187N
60000
50000
40000
30000
20000
10000
0
10 20 30 40 50 60 70 80 90

α2-6-linked linear and bi-antennary glycans (only 2 of 14 tested viruses bound to α2-3 sialosides) with no detectable differences in receptor specificity noted between swine viruses isolated from swine or humans, rationalizing the multiple interspecies transmission events documented since the 1970s (Dowdle, 1997; Gaydos et al., 1977; Myers et al., 2007, 2006). These findings are consistent with the reported similarity between the sialoglycans of swine respiratory epithelial cells and glycans involved in human influenza virus infection (Bateman et al., 2010). Furthermore, the repertoire of glycans recognized by swine viruses with classical HA was almost indistinguishable from that of 2009 pandemic H1N1 viruses, suggesting that the receptor specificity of swine influenza viruses circulating in the United States prior to the emergence of 2009 pandemic H1N1 was fit for sustained transmission in the human population.

The host cells supporting viral replication play a major role in selecting variant viruses with mutations in the globular head of HA, near to the receptor binding site. The majority of 2009 H1N1 pandemic viruses encode aspartic acid (D) at position 222 and glutamine (Q) at position 223 of HA, but variants with D222G (glycine) and Q223R (arginine) substitutions were found in egg-grown viruses (Robertson et al., 2011). Although these mutations alone do not seem to affect virus antigenicity by hemagglutination-inhibition (HI) (Robertson et al., 2011), viruses with the D222G change in HA have been associated with severe infection outcomes (Chen et al., 2010; Kilander et al., 2010; Potdar et al., 2010; WHO, 2010). The D222G viruses recognized a comparable set of α2-6 linked sialosides but in addition bound to a broad repertoire of α2-3-linked sialosides. Binding to a similar repertoire of α2-3 linked sialosides by viruses with D222G was recently reported (Liu et al., 2010). Specifically, Liu et al. reported binding of D222G 2009 H1N1 viruses to 10 α2-3-sialylglycans. Although the glycans in the CDC and Liu et al. (2010) arrays are not identical, nine structures in the two arrays can be considered structurally equivalent in at least the sialic acid and three adjacent hexoses. Two glycans differ only in glucose N-acetylation at the reducing end. Other glycans differ mainly by the presence of additional lactose and ceramide at the reducing end of glycans from the Liu et al. array. Nevertheless, six of the nine glycans considered similar in the two platforms were bound by the D222G viruses in both arrays (Supplementary Fig. 3 and Fig. 5A) (Liu et al., 2010). Two of the three glycans in the CDC array that were not bound by the D222G viruses lacked the lactose-ceramide extension at the reducing end of the Liu et al. (2010) glycans. In addition, virion densities loaded on the arrays were pre-optimized by titration for the generation of a subsaturating fluorescence signal with minimal binding to irrelevant glycans and standardized to an HA titer of 128 for all viruses. Considering that HA interactions with its ligands in glycan arrays tend to be very sensitive to experimental conditions, we interpret agreement in 6 of 9 glycan probes in the two array platforms as indicative of comparable receptor binding functions by the viruses in the two laboratories. Previous glycan microarray studies with D222G recombinant HA from pandemic 2009 H1N1 showed weak binding restricted to sulfated α2-3 sialylglycans as well as α2-3 and α2-3/α2-6 disialoside structures, probably due to the lower valency of the HA in this assay as compared to that of virions (Yang et al., 2010). Taken together, the increased binding of α2-3 sialosides by human 2009 H1N1 influenza viruses with D222G mutations might provide a rationale for its association with severe or fatal disease in humans. Nevertheless, such association could be biased by the collection of lower respiratory tract specimens from severely ill hospitalized influenza patients rather than nasopharyngeal samples from mild cases in outpatient health care settings (Kilander et al., 2010; Mak et al., 2010; Smith et al., 2010; WHO, 2010).

To investigate whether receptor specificity 'barriers' prevented the sustained transmission of swine H1N1 viruses among humans in previous decades, we analyzed the receptor binding profiles of classical swine influenza from 1945 onwards. The dual α2-3 and α2-6 receptor binding specificity of older classical viruses cannot be interpreted unambiguously due to the presence of the D222G substitution in HA, which may have been selected during isolation and propagation in eggs. In an attempt to mitigate the negative consequences of isolation in eggs, the A/Swine/Iowa/1945 and A/swine/Wisconsin/1/1968 viruses used in this study were re-isolated in MDCK cells from bronchoalveolar lavage fluid collected from pigs experimentally infected with the original egg isolate (Vincent et al., 2006). However, the postulated D222G substitution and concomitant change in receptor binding specificity did not appear to compromise viral fitness for swine, and reversion to D at position 222 was not observed. Due to the lack of archival clinical specimens or MDCK isolates, the actual amino acid at position 222 and the receptor specificity of genuine swine H1N1 viruses from the middle of the 20th century remains to be determined.

To investigate whether human seasonal influenza viruses would undergo specific changes in receptor binding specificity following sustained transmission in swine, we analyzed two 'human-like' H1N1 viruses from swine. A/swine/Minnesota/02011/2008 showed a slightly increased binding to α2-3 sialosides as compared to the ancestral A/New Caledonia/20/1999 human seasonal virus. In the case of A/swine/Iowa/02039/2008 virus, the receptor binding properties correlated with the presence of a D187N substitution in HA of this virus, in the absence of propagation in eggs. Position 187 is a critical structural determinant of HA receptor specificity among H1N1 influenza viruses. Previous studies have proposed that amino acid 187D in the 1918 pandemic H1N1 and A/swine/Iowa/1930 H1N1 viruses hydrogen bonds with GlcNAc of α2-6 sialosides and further stabilizes the glycan–receptor interaction (Gamblin et al., 2004; Stevens et al., 2006). A single mutation in a variant 1918 pandemic virus (D187E) was sufficient to revert its glycan binding preference towards α2-3-linked sialoside binding (Stevens et al., 2006). These data suggest that the 'human-like' influenza viruses that acquire transmissibility in swine populations retain the receptor repertoire of their human influenza virus ancestors and show early signs of binding some α2-3 sialosides.

Classical swine H1N1 viruses were reported to bind to gangliosides with N-glycolyl neuranimic acid (Suzuki et al., 1997). However, swine influenza viruses analyzed in this study did not bind to NeuGc sialoglycans on the array, in agreement with a previous report (Gambaryan et al., 2005). These findings might be explained by differences in the viral isolates used as well as the topology of the sialic acids in the glycan array, thin-layer chromatography, and soluble glycoprotein assay systems which might affect not only their interaction with HA but also the valency of virion binding (Chandrasekaran et al., 2008; Collins and Paulson, 2004).

In closing, it is important to point out that the 2009 pandemic H1N1 viruses share their preference for α2-6 sialoside receptors with seasonal human H1N1 viruses, and their receptor binding specificity was very similar to that of their ancestral swine viruses with a 'classical' swine HA. These findings suggest that the incorporation of divergent Eurasian genes, such as NA and M, merit further investigation to determine their possible role in the acquisition of human transmissibility. It is possible that their concerted activity with the HA and other genes from North American swine might have contributed key functional properties that enabled the emergence and global spread of the new 2009 pandemic H1N1 virus.

Materials and methods

Viruses and cells

Viruses selected for this study are listed in Table 1. Virus propagation in Madin-Darby canine kidney (MDCK) cells was performed in Dulbecco's Modification of Eagle's Medium supplemented with 1 μg/ml TPCK-treated trypsin. Virus propagation in eggs was

performed as described previously (Szretter et al., 2006). Approximately 10 EID_{50} of virus harvested from eggs were used for glycan array analysis. The HA genes of all virus stocks used in this study were sequenced to detect the emergence of sequence variants and quasispecies during growth and amplification.

Reverse genetics

Original, mutant or reassortant viruses were generated from plasmids by a reverse genetics approach. Viral cDNAs were cloned into a dual promoter plasmid vector (Hoffmann et al., 2000). To generate viruses with amino acid changes in the HA, mutations were introduced into the plasmid using QuikChange site directed mutagenesis system from Stratagene (CA). Viruses were rescued by plasmid transfection of HK293 cells, followed by propagation in MDCK or eggs. The HA gene of resulting virus stocks were sequenced to detect the emergence of possible revertants and subdominant sequence variants during amplification.

Glycan microarray analysis

Analysis of the receptor specificity of influenza virus using glycan microarrays was done largely as described previously (Blixt et al., 2004; Stevens et al., 2006) Custom arrays for influenza research were produced for the CDC on NHS activated glass slides (Schott Nexterion, Mainz, Germany) using a glycan library provided by the Consortium for Functional Glycomics (http://www.functionalglycomics.org; See Table S1 for list of glycan structures). Virus preparations were diluted to 1 mL into phosphate buffered saline buffer containing 3% (w/v) bovine serum albumin (PBS-BSA) to HA titers of 128. Virus suspensions were applied to slides and the slides were incubated in a closed container and subjected to gentle agitation at room temperature for 1 h in the presence of 10 or 300 nM Zanamivir. Unbound virus was washed off by dipping slides sequentially in PBS. The slides were then overlaid with corresponding primary antibodies (sheep anti-A /California/07/2009, A/New Caledonia/20/1999, swine anti- A/swine/Iowa/1945 or A/swine/Wisconsin/1/1968) diluted in PBS-BSA, followed by incubation with biotinylated secondary antibody for 30 min. Slides were washed briefly with PBS as described earlier followed by application of the avidin-Alexa fluro 635 conjugates. After drying the slides in a steam of nitrogen they were scanned (ProScanArray HT slide scanner, Perkin Elmer or Genepix Molecular Device) followed by image analysis with ImaGene 6.1 software (Biodiscovery, Inc., El Segundo, CA).

Supplementary materials related to this article can be found online at doi: 10.1016/j.virol.2011.01.015.

Acknowledgments

Glycan microarrays were produced for the Centers for Disease Control using the glycan library of CFG developed under funding by the National Institute of General Medical Sciences, Grant GM62116. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry.

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