2013

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Virology 446 (2013) 225–229

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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ARTICLE INFO

Article history:
Received 6 May 2013
Returned to author for revisions
30 May 2013
Accepted 6 August 2013
Available online 4 September 2013

Keywords:
Antigenic drift
H3N2
Influenza A virus
R189K
H3N2v
Antigenic variant
Antigenic cartography
Serological assay

ABSTRACT

Two distinct antigenic clusters were previously identified among the H3N2 swine influenza A viruses (IAVs) and were designated H3N2SIV-alpha and H3N2SIV-beta (Feng et al., 2013. Journal of Virology 87 (13), 7655–7667). A consistent mutation was observed at the position 189 of hemagglutinin (R189K) between H3N2SIV-alpha and H3N2SIV-beta fair isolates. To evaluate the contribution of R189K mutation to the antigenic drift from H3N2SIV-alpha to H3N2SIV-beta, four reassortant viruses with 189R or 189K were generated. The antigenic cartography demonstrated that the R189K mutation in the hemagglutinin of H3N2 IAV contributed to the antigenic drift, separating these viruses into H3N2SIV-alpha to H3N2SIV-beta. This R189K mutation was also found to contribute to the cross-reaction with several ferret sera raised against historical human IAVs with hemagglutinin carrying 189K. This study suggests that the R189K mutation plays a vital role in the antigenicity of swine and human H3N2 IAVs and identification of this antigenic determinant will help us rapidly identify antigenic variants in influenza surveillance.

Introduction

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae*. Among the 17 HA subtypes of IAVs identified, H3 is one of the most widely circulating subtypes in nature. H3 IAVs have been recovered from humans, pigs, horses, dogs, birds, and seals. H3 IAVs caused the 1968 pandemic (by H3N2 IAV), contemporary seasonal epidemics (H3N2) in humans, epidemic or endemic diseases in pigs (H3N2) (Zhou et al., 1999, 2000), horses (H3N8) (Thomson et al., 1977), and dogs (H3N2 and H3N8) (Crawford et al., 2005; Li et al., 2010; Song et al., 2008; Yoon et al., 2005).

In the North American swine population, the current predominant H3N2 IAV was associated with a "spillover" of human seasonal H3N2 IAVs to pigs in 1990's (Vincent et al., 2008; Zhou et al., 1999). Phylogenetic analyses of HA genes of H3N2 SIVs in North America demonstrated that there have been at least four genetic groups (Cluster I–IV) (Olsen et al., 2006), and H3N2 IAVs of Cluster IV has predominated in US swine populations since 2005 (Hause et al., 2010). Neutralization assay using swine antisera demonstrated that these four genetic clusters are also antigenically distinct, varying from a 2 to 8-fold change in hemagglutination inhibition (HI) titers, although cross reaction exists among these clusters to a degree (Hause et al., 2010).

In 2011, a novel IAV, so called H3N2 variant (H3N2v), was identified in agricultural fairs. This virus caused more than 325 confirmed human influenza cases in 14 states (CDC, 2012a, 2012b; Lindstrom et al., 2012). Genetically, the hemagglutinin gene of H3N2v-like IAV belongs to Cluster IV of H3N2 SIVs. Recently, antigenic profile of four human H3N2v isolates, 12 commercial swine farm isolates, and 68 isolates recovered from pigs at 2009–2011 Ohio county fairs were characterized in our laboratory (Feng et al., 2013). These 84 isolates were clearly divided into two antigenic clusters, H3N2SIV-alpha and H3N2SIV-beta. The human H3N2v isolates were grouped with H3N2 SIV-beta while the swine isolates were divided between two antigenic clusters. Sequence analysis of these isolates showed a number of variations at antibody binding sites among these H3N2 isolates, but only the

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0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.08.004

mutation arginine (R) to lysine (K) at the position 189 of hemagglutinin was consistent between H3N2SIV-alpha and H3N2SIV-beta. Also, our previous study showed that the viruses in the antigenic cluster H3N2SIV-beta cross-reacted with ferret antisera produced against several seasonal human influenza viruses (Feng et al., 2013). Interestingly, these human seasonal viruses also carried 189K in HA.

In this study, four reassortants with 189R or 189K were generated by reverse genetics, and serological assays were conducted for these reassortants to determine if the R189K mutation drives the antigenic drift of these H3N2 SIVs. In addition, this study was conducted to determine if the R189K mutation contributes to the cross-reaction to sera against human seasonal viruses.

Results and discussion

To evaluate contribution of R189K mutation to antigenic drift of H3N2 IAVs from Ohio county fairs, four reassortant viruses were generated by reverse genetics and designated as 09SW64_189R (wt), 09SW64_189K (mt), 11SW347_189K (wt) and 11SW347_189R (mt) (Table 1). The 09SW64_189R (wt) represented an IAV in the antigenic cluster H3N2SIV-alpha whereas 11SW347_189K (wt) represented an IAV in the antigenic cluster H3N2SIV-beta. The HI results showed that the antisera against H3N2SIV-alpha isolates had a HI titer of 1280 to 2560 against 09SW64_189R (wt) whereas the antisera against H3N2SIV-beta isolates had a HI of 40–80 against 09SW64_189R (wt). In contrast, the antisera against H3N2SIV-beta isolates had a HI titer of 480–960 against 11SW347_189K (wt) whereas the antisera against H3N2SIV-alpha isolates had a HI of 10–20 against 11SW347_189K (wt). The antigenic profile of these two reassortant viruses resembled their wild type isolates (A/swine/Ohio/09SW64/2009(H3N2) and A/swine/Ohio/11SW347/2011 (H3N2), respectively. Antigenic cartography confirmed each of these two reassortant viruses was located in the corresponding antigenic cluster: 09SW64_189R (wt) belonged to H3N2SIV-alpha whereas 11SW347_189K (wt) was clustered to H3N2SIV-beta (Fig. 1).

The antisera against H3N2SIV-alpha isolates had HI titers ranging from 640 to 1280 to 09SW64_189K (mt), and these HI titers were two folds lower than those HI titers for the same set of antisera to 09SW64_189R (wt); the antisera against H3N2SIV-beta isolates had HI titers ranging from 240 to 320 to 09SW64_189K (mt), and these HI titers were at least four-fold higher than those HI titers for the same set of antisera against 09SW64_189R (wt) (Table 1). On the other hand, the antisera against H3N2SIV-alpha isolates had HI titers ranging from 20 to 80 to 11SW347_189R (mt), and these HI titers were at least two folds higher than those HI titers for the same set of antisera to 11SW347_189K (wt); the antisera against H3N2SIV-beta isolates had HI titers ranging from 120 to 320 to 11SW347_189R (mt), and these HI titers were at least three folds lower than those HI titers for the same set of antisera to 11SW347_189K (wt). These HI data demonstrate that the R189K mutation in the hemagglutinin of H3N2 IAV contributed to the antigenic drift. Antigenic cartography demonstrated that the R189K mutation could move A/swine/09SW64/2009 (H3N2) from antigenic cluster H3N2SIV-alpha forward to antigenic cluster H3N2SIV-beta whereas the K189R mutation could drive A/swine/11SW347/2011(H3N2) from antigenic cluster H3N2SIV-beta toward antigenic cluster H3N2SIV-alpha (Fig. 1).

In this study, we also produced ferret antisera against the two mutant strains 09SW64_189K and 11SW347_189R. The HI assay demonstrated the antisera against 09SW64_189K showed 2-fold higher HI titer for 11SW347_189K than 11SW347_189R, and the antisera against 11SW347_189R had 4-fold higher HI titer for 09SW64_189R than 09SW64_189K (Table 1). This data further supported our conclusion that the single R189K mutation in the HA of swine H3N2 IAV contributed to the antigenic drift, separating these viruses into H3N2SIV-alpha to H3N2SIV-beta.

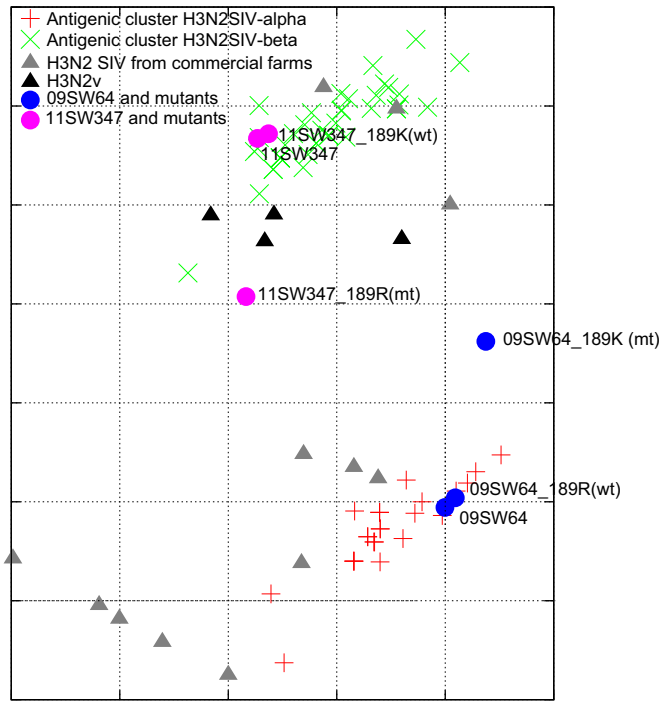

Fig. 1. Antigenic cartography was constructed using HI data for 68 SIV isolates from Ohio agricultural fairs (2009–2011),12 SIV isolates from commercial farms (2006–2012), and four human H3N2v influenza virus isolates, and four reassortant viruses (09SW64_189R, 09SW64_189K, 11SW347_189R, and 11SW347_189K). The HI data for 80 SIV isolates and four human H3N2v isolates were adapted from (Feng et al., 2013), and those for four reassortant viruses were shown in Table 1. Antigenic clusters H3N2SIV-alpha and H3N2SIV-beta and the viruses in each clusters were described in (Feng et al., 2013). 09SW64 and the corresponding reassortants (09SW64_189R and 09SW64_189K) were marked in blue whereas 11SW347 and the corresponding reassortants (11SW347_189R and 11SW347_189K) in pink. This cartography demonstrates that R189K moves 09SW64 from antigenic cluster H3N2SIV-alpha towards H3N2SIV-beta and that K189R moves 11SW347 from antigenic cluster H3N2SIV-beta towards H3N2SIV-alpha. Antigenic cartography was constructed using AntigenMap (http://sysbio.cvm.msstate.edu/AntigenMap) (Barnett et al., 2012; Cai et al., 2010).

Our results also suggested that the changes in heterologous titers were more than homologous titers. For example, the changes in 11SW347_189K and 11SW347_189R against their corresponding antibody were about two fold, whereas those in 10SW215 against 11SW347_189K and 11SW347_189R ferret antisera were four fold. This indicated that some other mutations (not in reported antibody binding sites of H3N2 IAVs(Wilson and Cox, 1990)) in HA, NA, or MP protein might affect influenza antigenicity indirectly, although R189K is the predominant mutation leading to antigenic drift in H3N2 SIVs.

Our previous study showed that the viruses in the antigenic cluster H3N2SIV-beta cross-reacted with ferret antisera produced against three seasonal human influenza viruses, including A/Caen/1/1984(H3N2), A/Ann Arbor/03/1993(H3N2), and A/Nanchang/933/1995(H3N2). Among these, the ferret antiserum against A/Caen/1/1984(H3N2) exhibited the highest HI titer, 1:160, to the viruses in the antigenic cluster H3N2SIV-beta whereas the ferret antiserum against A/Caen/1/1984(H3N2) does not have any cross reactions against the viruses in the antigenic cluster H3N2SIV-alpha (Feng et al., 2013). Sequence analysis showed that hemagglutinin of A/Caen/1/1984(H3N2) also carried 189K, like the hemagglutinin of IAV in the antigenic cluster H3N2SIV-beta. To evaluate the contribution of the R189K mutation in that cross-reaction, HI assay was performed on the four reassortant viruses using antisera produced against several historic seasonal human

influenza virus strains with or without 189K. As described in Table 2, the HI titers for the ferret antisera against A/Caen/1/1984 (H3N2), A/Mississippi/1/1985(H3N2), and A/Leningrad/360/1986 (H3N2), all of which had 189K, had higher HI titers to those reassortant viruses carrying 189K than to those carrying 189R. Although the ferret antiserum against A/Philippines/2/1982 (H3N2) did not show cross reaction with 09SW64_189K, this serum had a titer of 20 against 11SW347_189K but did not cross react with 11SW347_189R. These results suggest that position 189 would be an important antibody binding site, as is consistent with previous antigenic analysis of human seasonal H3N2 IAV (Huang et al., 2009; Smith et al., 2004). It was recently reported that K189N/D/Q/E mutations were involved in the antigenic drift of horse H3N8 isolates collected between 1968 and 2007 (Lewis et al., 2011). These observations further highlight the important role of amino acid at position 189 in the antigenicity of H3 IAVs.

Compared with those isolates in antigenic cluster H3N2SIV_alpha, the H3N2 swine influenza isolates in H3N2SIV_beta have two major differences: (1) the introduction of matrix gene from 2009 H1N1 influenza A virus; (2) a consistent mutation R189K, which is located in the 190-loop of receptor binding site in H3 subtype of influenza A virus. Previous study demonstrated that introduction of M gene from 2009 pandemic virus increased viral transmissibility in ferrets (Pearce et al., 2012). However, the R189K seemed not to affect much receptor binding profiles based on the results on six glycans (Neu5Acα2–3Galβ1–4GlcNAcβ1-; Neu5Acα2–6Galβ1–4GlcNAcβ1-; Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1-; Neu5Acα2–6Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1-; and Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1-) (Pearce et al., 2012). Because these glycans might not represent the entire glycan profile in human respiratory system, it would be interesting to characterize the role of R189K in transmission of H3N2v from swine to human.

To reduce the risks of human infection of H3N2v virus, one option is to vaccinate these pigs to reduce their viral shedding before sending them to county fairs. Thus, it will be critical to perform influenza surveillance and understand both antigenic and genetic dynamics of these viruses in the county fair pigs as well as those pigs to be deployed to county fairs. Identification of antigenic variants is one of the keys to a successful vaccination program. This study suggested that 189K be an important biomarker for the viruses in H3N2SIV_beta. The viruses in H3N2SIV_beta are predominant in county fairs and antigenically similar to human H3N2v isolates but different from the viruses in H3N2SIV_alpha, which are also circulating in swine population (Feng et al., 2013).

Conclusions

In summary, we experimentally demonstrated that a single mutation (i.e., R189K) in hemagglutinin contributed to the antigenic drift of contemporary H3N2 SIVs. To our knowledge, it is the first study to demonstrate contribution of R189K mutation to antigenic drift among swine H3N2 isolates. Identification of this antigenic determinant will help us rapidly identify antigenic variants in influenza surveillance, especially those with an antigenic profile similar to the emerging human H3N2v IAVs.

Materials and methods

Cells and viruses

Madin-Darby Canine Kidney cells (MDCK) and human embryonic kidney cells (293T) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Both cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO/BRL, Grand Island, NY), supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), penicillin–streptomycin, and amphotericin B (GIBCO/BRL, Grand Island, NY), at 37 °C with 5% CO_2. All the viruses generated by reverse genetics were propagated in MDCK cells cultured in Opti-MEM medium (GIBCO/BRL, Grand Island, NY) supplemented with 1 μg/ml TPCK-Trypsin (Sigma-Aldrich, St. Louis, MO), penicillin–streptomycin, and amphotericin B (GIBCO/BRL, Grand Island, NY), at 37 °C with 5% CO_2.

Plasmids and site directed mutagenesis

The eight gene segments of PR8 were kindly provided by Dr. Richard Webby at St. Jude Children's Research Hospital. The hemagglutinin gene of A/swine/Ohio/09SW0964/2009 (H3N2) (09SW64) (GenBank accession number: CY130717.1) and A/swine/Ohio/11SW347/2011(H3N2) (11SW347) (GenBank accession number: CY131957.1) and the neuraminidase gene from A/Sydney/05/1997 (H3N2) (SY97) were cloned into pHW2000 vector as described elsewhere (Cai et al., 2012). The R189K in hemagglutinin of 09SW64 and K189R in hemagglutinin of 11SW347 were introduced using the QuickChange II site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) according to manufacturer's protocols. The presence of the corresponding mutation (without any other unintended mutations) was confirmed by sequencing.

Generation of reassortant viruses by reverse genetics

The reassortant viruses were recovered by transfection in co-cultured 293T and MDCK cells in 6-well plates. Briefly, 1 μg of each of six plasmids, each of which carries one of six PR8 internal gene (polymerase PB1, polymerase PA, polymerase PB2, nucleoprotein gene, matrix gene, or nonstructural gene), were mixed with 1 μg of SY97 neuraminidase plus 1 μg of 09SW64, 11SW347, 09SW64-189K, or 11SW347-189R hemagglutinin gene in 234 μl Opti-MEM medium. Then, 16 μl of TransIT®-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI) was added into the mixture. The mixture was incubated at ambient temperature for 45 min, and then 800 μl of Opti-MEM medium was added and mixed. The mixture was then added to the co-cultured 293T and MDCK cells in 6-well plates. Twelve hours post transfection, the medium was changed with 2 ml of fresh Opti-MEM medium with 1 μg/ml TPCK-Trypsin (Sigma-Aldrich, St. Louis, MO). At day 3 post transfection, a half of the medium plus cells were inoculated into MDCK cell. Ninety-six hours post inoculation, the supernatant from the infected cells was collected and stored in −80 °C until use. As a result, four 6+2 reassortants were generated, so called 09SW64_189R, 09SW64_189K, 11SW347_189K and 11SW347_189R (Table 1).

Genomic sequencing

The vRNA and cDNA were prepared as previously described (Ye et al., 2010). Eight fragments were amplified and sequenced using a combination of universal and custom made primers (available upon request) (Hoffmann et al., 2001). Sequencing was performed by Life Sciences Core Laboratories Center at Cornell University using the Applied Biosystems Automated 3730 DNA Analyzer, which utilized Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase.

Generation of ferret antisera

Ferret antisera were generated in 6- to 8-week-old ferrets, which had baseline HI titers less than 1:10 against A/Brisbane/10/2007

Table 1
Antigenic characterization of H3N2 reassortant viruses using hemagglutination inhibition assay against ferret antisera for H3N2 swine influenza viruses.

Virus[a]	Antigenic cluster[d]	Ferret antisera[f]								
		09SW64 (189R)[e]	09SW64[c] (189K)	09SW96 (189R)	10SW130 (189K)	10SW156 (189K)	10SW215 (189K)	11SW208 (189K)	11SW347 (189K)	11SW347[c] (189R)
09SW64[b]	H3N2SIV-alpha	**2560**	1280	1280	80	80	80	60	40	320
09SW64_189R (wt)[c]		2560	1920	1280	80	80	80	80	40	480
09SW64_189K[c]		1280	**1920**	640	320	320	320	320	240	120
11SW347[b]	H3N2SIV-beta	20	ND[g]	10	320	480	480	1280	**960**	ND
11SW347_189K (wt)[c]		20	80	< 10	480	480	640	960	640	640
11SW347_189R[c]		80	40	20	120	120	120	320	240	**1280**

[a] 09SW64, A/swine/Ohio/09SW0964/2009(H3N2); 09SW96, A/swine/Ohio/09SW96/2009(H3N2); 10SW130, A/swine/Ohio/10SW130/2010(H3N2); 10SW156, A/swine/Ohio/10SW156/2010(H3N2); 10SW215, A/swine/Ohio/10SW215/2010(H3N2); 11SW208, A/swine/Ohio/11SW208/2011(H3N2); 11SW347, A/swine/Ohio/11SW347/2011(H3N2).
[b] 09SW64 and 11SW347 are wild type strains used to generate reassortant viruses.
[c] Reassortant viruses with HA gene from either 09SW64 or 11SW347, NA gene from A/Sydney/05/1997 (H3N2), and six internal genes from A/PR/8/1934(H1N1).
[d] Antigenic clusters were defined by antigenic cartography using *k*-mean clustering method in (Feng et al., 2013).
[e] The amino acid at the position 189 of hemagglutinin of the corresponding virus.
[f] The number in bold is the homologous HI title.
[g] ND, not done.

Table 2
Antigenic characterization of H3N2 reassortant viruses using hemagglutination inhibition assay against ferret antisera for human seasonal influenza A viruses.

Virus[a]	Ferret antisera								
	PH82 (189K)[d]	CE84 (189K)	MS85 (189K)	LG86 (189K)	SC89 (189R)	AN93 (189S)	JO94 (189S)	NC95 (189S)	SY97 (189S)
09SW64[b]	< 10	20	< 10	< 10	< 10	20	10	20	< 10
09SW64_189R(wt)[c]	< 10	< 10	< 10	< 10	< 10	20	< 10	20	< 10
09SW64_189K[c]	< 10	160	40	10	< 10	20	< 10	20	< 10
11SW347[b]	10	80	10	10	10	40	< 10	10	< 10
11SW347_189K(wt)[c]	20	160	20	10	10	20	< 10	< 10	< 10
11SW347_189R[c]	< 10	40	< 10	< 10	10	20	< 10	< 10	< 10

[a] 09SW64, A/swine/Ohio/09SW0964/2009(H3N2); 09SW96, A/swine/Ohio/09SW96/2009(H3N2); 11SW347, A/swine/Ohio/11SW347/2011(H3N2); PH82, A/Philippine/2/82 (H3N2); CE84, A/Caen/1/1984(H3N2); MS85, A/Mississippi/1/1985(H3N2); LG86, A/Leningrad/360/1986(H3N2); SC89,A/Sichuan/60/1989 (H3N2); AN93, A/Ann Arbor/03/1993 (H3N2); JO94, A/Johannesburg/33/1994 (H3N2); NC95, A/Nanchang/933/1995 (H3N2); SY97, A/Sydney/05/1997 (H3N2).
[b] 09SW64 and 11SW347 are wild type strains used to generate reassortant viruses.
[c] Reassortant viruses with HA gene from either 09SW64 or 11SW347, NA gene from A/Sydney/05/1997 (H3N2), and six internal genes from A/PR/8/1934(H1N1).
[d] The amino acid at the position 189 of hemagglutinin of the corresponding virus.

(H3N2), A/Brisbane/59/2007(H1N1), and A/California/4/2009(H1N1). Each ferret was inoculated intranasally with 10^6 $TCID_{50}$ for mutant strain 09SW64_189K and 11SW347_189R respectively according to protocol IACUC-11-027 and IBC-011-11 approved by Office of Regulatory Compliance Institutional Biosafety Committee and Institutional Animal Care and Use Committee from Mississippi State University. Sera were collected from the ferrets at three weeks post inoculation if the HI titer in a ferret was at least 1:160 at 2 weeks post inoculation. Otherwise, a second dose of 10^6 $TCID_{50}$ of the corresponding virus was given and serum was collected five weeks post first inoculation. All sera were aliquoted and stored at -70° C until use.

In addition to the above two ferret sera, this study also included seven ferret antisera prepared against two H3N2 isolates in antigenic cluster H3N2SIV-alpha, A/swine/Ohio/09SW64/2009 (H3N2) and A/swine/Ohio/09SW96/2009(H3N2) and five isolates in antigenic cluster H3N2SIV-beta, A/swine/Ohio/10SW130/2010 (H3N2), A/swine/Ohio/10SW156/2010(H3N2), A/swine/Ohio/10SW215/2010(H3N2), A/swine/Ohio/11SW208/2011(H3N2) and A/swine/Ohio/11SW347/2011(H3N2). The generation of these sera was described in (Feng et al., 2013). In addition, nine ferret sera against human seasonal influenza viruses, A/Philippine/2/82 (H3N2), A/Caen/1/1984(H3N2), A/Mississippi/1/1985(H3N2), A/Leningrad/360/1986(H3N2), A/Sichuan/60/1989 (H3N2), A/Ann Arbor/03/1993 (H3N2), A/Johannesburg/33/1994 (H3N2), A/Nanchang/933/1995 (H3N2), and A/Sydney/05/1997 (H3N2) were also included, and the generation of these sera were described in (Sun et al. 2013). These sera are listed in Tables 1 and 2.

Hemagglutination (HA) and HI assays

Virus HA titers were measured using 0.5% turkey red blood cells according to the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/whocdscsrncs20025rev.pdf). In the HI assays, the antisera against different viruses listed in Tables 1 and 2 were first treated with receptor-destroying enzyme (RDE) (Denka Seiken Co., Tokyo, Japan) in 1:3 (volume of serum: volume of RDE) at 37 °C for 18 h, and then heat inactivated at 56 °C for 30 min. The sera were then diluted with phosphate-buffered saline (for a final dilution of 1:10). The anti-viral antibody titers were assessed using the HI assay outlined by the WHO Manual on Animal Influenza Diagnosis and Surveillance. Each HI was repeated at least three times, and the average value was documented (Tables 1 and 2).

Antigenic cartography

Antigenic mapping presents an intuitive way to visualize the antigenic relationship embedded in serological data by projecting antigens into a two or three dimensional map, and a larger antigenic discrepancy in serological data will be reflected as a larger distance in antigenic map (Cai et al., 2010, 2011). In this study, the antigenic map was generated based on HI data from 88 SIVs (68 fair SIV isolates, 4 human H3N2v isolates, 12 farm SIV isolates, and 4 reassortant viruses generated in this study) versus seven reference ferret antisera using AntigenMap by setting HI titers of 10 or less as low reactors (Barnett et al., 2012; Cai et al., 2010). Those HI data for 68 fair SIV isolates, 4 human H3N2v isolates, and 12 farm SIV isolates were adapted from (Feng et al., 2013). Data normalization was performed as described elsewhere (Cai et al., 2012).

Acknowledgments

We are grateful for Dr. Zhixin Feng for his assistance in ferret sera generation. This work was supported in part by the Minnesota Center of Excellence for Influenza Research and Surveillance (MCEIRS) with federal funds from the Centers of Excellence for Influenza Research and Surveillance (CEIRS), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract no. HHSN266200700007C. The finding and conclusions in this study are those of the authors and do not necessarily reflect the views of the funding agency.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.08.004.

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