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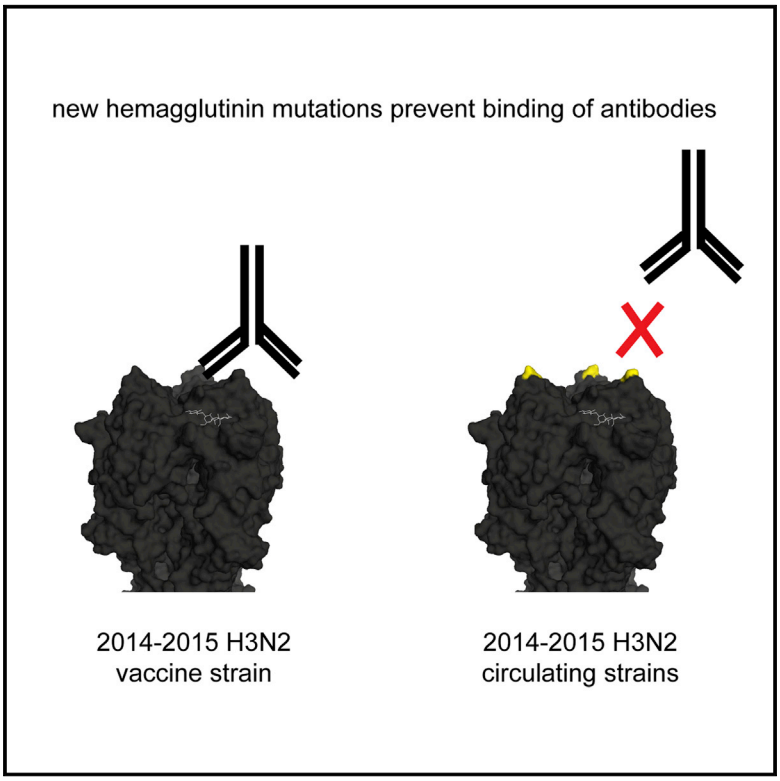
Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014–2015 Influenza Season

Graphical Abstract



Highlights

- Recent H3N2 strains are antigenically distinct in comparison to the 2014–2015 vaccine strain
- Most humans produce antigenic site B HA antibodies
- New mutations in antigenic site B of HA likely led to 2014–2015 vaccine mismatch

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In Brief

Most H3N2 influenza viruses circulating during the 2014–2015 influenza season were antigenically mismatched to the H3N2 component of the 2014–2015 influenza vaccine. Chambers et al. use reverse genetics to identify the hemagglutinin mutations responsible for this antigenic mismatch.

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Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014–2015 Influenza Season

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SUMMARY

Influenza vaccines must be updated regularly because influenza viruses continuously acquire mutations in antibody binding sites of hemagglutinin (HA). The majority of H3N2 strains circulating in the Northern Hemisphere during the 2014–2015 season are antigenically mismatched to the A/Texas/50/2012 H3N2 vaccine strain. Recent H3N2 strains possess several new HA mutations, and it is unknown which of these mutations contribute to the 2014–2015 vaccine mismatch. Here, we use reverse genetics to demonstrate that mutations in HA antigenic site B are primarily responsible for the current mismatch. Sera isolated from vaccinated humans and infected ferrets and sheep had reduced hemagglutination inhibition and in vitro neutralization titers against reverse-genetics-derived viruses possessing mutations in the HA antigenic site B. These data provide an antigenic explanation for the low influenza vaccine efficacy observed during the 2014–2015 influenza season. Furthermore, our data support the World Health Organization's decision to update the H3N2 component of future vaccine formulations.

INTRODUCTION

Most neutralizing influenza antibodies (Abs) target the hemagglutinin (HA) glycoprotein. Seasonal influenza vaccines are designed to elicit HA Abs; however, these vaccines are ineffective when viruses acquire mutations in HA Ab binding sites (Yewdell, 2011). Mid-season influenza vaccine efficiency rates during the 2014–2015 Northern Hemisphere season are extremely low (Flannery et al., 2015; Pebody et al., 2015), and recent H3N2 strains are antigenically distinct compared to the 2014–2015 A/Texas/50/2012 H3N2 vaccine strain (D'Mello et al., 2015). The 2014–2015 H3N2 strains can be grouped into at least three genetically distinct clades (Broberg et al., 2015). Viruses within each genetic clade possess several shared and unique HA mutations, and it is currently unclear which of these mutations are antigenically relevant.

H3 HAs have at least five distinct antigenic sites (sites A–E) (Wiley et al., 1981). Seasonal influenza vaccine strains are routinely chosen based on antigenic analyses that utilize antisera prepared in ferrets (Stöhr et al., 2012). Koel and colleagues recently demonstrated that most primary ferret Ab responses to H3N2 viruses are heavily focused on H3 antigenic sites A and B (Koel et al., 2013). Our studies and others have demonstrated that prior H1N1 influenza exposures can influence the specificity of Ab responses raised against new H1N1 influenza strains (Hensley, 2014; Li et al., 2013b; Linderman et al., 2014). We found that ferret antisera do not always recapitulate the different types of H1N1 Ab specificities that are found in individual humans with vastly different pre-exposure histories. Human Ab responses appear to be focused on antigenic site A of some H3 strains (Abe et al., 2004) and on antigenic site B of other H3 strains (Popova et al., 2012).

It is important to determine which HA residues are responsible for the observed antigenic drift of 2014–2015 H3N2 strains. This information can be useful for guiding the selection of viral strains for future vaccine formulations. Here, we completed serological assays using A/Texas/50/2012 H3N2 viruses engineered to have specific HA mutations that are present in currently circulating H3N2 strains. We find that mutations in H3 antigenic site B significantly decrease the binding of ferret, sheep, and human Abs elicited by the A/Texas/50/2012 H3N2 vaccine strain. The World Health Organization recently recommended that the H3 component of seasonal influenza vaccines should be updated to include A/Switzerland/9715293/2013-like strains (Anonymous, 2015). Our data support this recommendation, although we note that the majority of currently circulating H3N2 strains have a distinct antigenic site B compared to the A/Switzerland/9715293/2013 strain.

RESULTS

2014–2015 H3N2 Viruses Possess Several HA Mutations

The H3N2 component of the 2014–2015 influenza vaccine is A/Texas/50/2012, which belongs to the 3C.1 HA genetic clade (Broberg et al., 2015). During the 2014–2015 season, H3N2

Table 1. HA Mutations in 2014–2015 H3N2 Viruses

Clades		
3C.2a	3C.3	3C.3a
L3I		
	T128A	T128A
		A138S
	R142G	R142G
N144S		
N145S	N145S	N145S
F159Y		F159S
K160T		
N225D		N225D
Q311H		

Shown are HA residues (H3 numbering) that differ between the A/Texas/50/2012 H3N2 vaccine strain and most clade 3C.2a, 3C.3, and 3C.3a viruses isolated during the 2014–2015 Northern Hemisphere influenza season.

strains belonging to the phylogenetic 3C.2a, 3C.3, and 3C.3a HA clades predominated (Broberg et al., 2015). Compared to the A/Texas/50/2012 strain, 3C.2a viruses possess HA differences at L3I, N144S, N145S, F159Y, K160T, N225D, and Q311H, 3C.3 viruses possess HA differences at T128A, R142G, and N145S, and 3C.3a viruses possess HA differences at T128A, A138S, R142G, N145S, F159S, and N225D (Table 1). HA clade 3C.2a and 3C.3a viruses are antigenically distinct compared to the A/Texas/50/2012 strain and the World Health Organization has recommended that the H3N2 component should be updated with an A/Switzerland/9715293/2013-like (HA 3C.3a) virus for the Northern Hemisphere 2015–2016 vaccine (Anonymous, 2015).

Analyses of HA sequences deposited in the GISAID database revealed that the majority of H3N2 viruses circulating in the United States in the fall and winter of 2014 belong to HA clade 3C.2a (Figure 1A). To understand the antigenic basis for the extremely poor H3N2 vaccine match during the 2014–2015 influenza season, we created a panel of A/Texas/50/2012 viruses that possessed HA mutations found in clade 3C.2a, 3C.3, and 3C.3a viruses (Table S1), and we completed antigenic analyses using sera isolated from ferret, sheep, and humans exposed to the A/Texas/50/2012 H3N2 vaccine strain (Tables 2 and 3). Our mutant viral panel includes every mutation from Table 1 that is located in the HA globular head (Figure 1B), with the exception of the F159Y and K160T mutations. We were able to successfully rescue A/Texas/50/2012 viruses that possessed the F159Y and K160T mutations, but these viruses failed to agglutinate red blood cells and quickly mutated when propagated for antigenic analyses. However, we were still able to assess the antigenic relevance of mutations in this area of HA since our mutant panel included a virus with the F159S HA mutation.

Ferrets and Sheep Infected with A/Texas/50/2012 Mount Abs against HA Antigenic Site B

We first completed hemagglutination-inhibition (HAI) assays with our mutant A/Texas/50/2012 virus panel and sera collected from ferrets and sheep recovering from A/Texas/50/2012 infections (Table 2). As expected, anti-A/Texas/50/2012 Ab titers were drastically decreased using the antigenically distinct A/Switzerland/9715293/2013 clade 3C.3a virus. Strikingly, anti-A/Texas/50/2012 Ab titers were decreased 4-fold in HAI assays with viruses engineered to possess a single F159S HA mutation (Table 2). Residue 159 is located in a highly exposed region of antigenic site B of H3 HAs (Figure 1B). Other HA mutations had more subtle effects on anti-A/Texas/50/2012 HAI titers. For example, the A138S and N145S HA mutations each lead to 2-fold decreases in HAI titers. We previously showed that viruses that bind to red blood cells with a high avidity can escape Abs in HAI assays independently of antigenic change (Li et al., 2013a). We found that A138S and N145S mutant viruses bound to red blood cells with a higher avidity (Table S1), and the apparent decreased HAI titers using these viruses likely result from this increase in receptor binding avidity. We found that viruses possessing the F159S mutation bound to red blood cells with a decreased avidity (Table S1), and for this reason, we concluded that reduced HAI titers using this virus were the result of a genuine antigenic change.

To verify the antigenic relevance of the F159S HA mutation, we completed additional direct Ab ELISA binding assays with plates coated with viral like particles (VLPs) possessing A/Texas/50/2012-WT HA or A/Texas/50/2012-F159S HA (Figure 1C). As a control, we coated plates with VLPs possessing the antigenically distinct A/Port Chalmers/1/1973 HA (from a 1973 H3 virus). Anti-sera isolated from ferrets infected with A/Texas/50/2012 had reduced binding to VLPs possessing A/Texas/50/2012-F159S HA compared to VLPs possessing A/Texas/50/2012-WT HA (Figure 1C). We verified that equal amounts of VLPs were used in these assays by completing additional ELISA experiments with the F49 monoclonal antibody (mAb) that recognizes the conserved stalk region of H3 (Figure 1D). Collectively, these studies indicate that ferrets and sheep mount Ab responses against an HA epitope involving residue F159 following infection with A/Texas/50/2012, and for this reason we focused the rest of antigenic analyses on this region of HA.

Ferrets and Sheep Infected with A/Switzerland/9715293/2013 Mount Ab Responses that Are Not Focused against HA Epitope Involving Residue 159

We also completed HAI assays using our mutant viral panel and anti-sera isolated from sheep infected with A/Switzerland/9715293/2013 virus. Anti-sera isolated from A/Switzerland/9715293/2013-infected animals reacted to all mutant A/Texas/50/2012 viruses, including viruses that possessed the site B F159S HA mutation (Table 2). Therefore, the F159S HA mutation results in an asymmetrical antigenic change. These data are important since A/Switzerland/9715293/2013 (an HA clade 3C.3a virus) has been chosen as the H3 component of 2015–2016 seasonal vaccines, even though HA clade 3C.2a viruses predominated toward the end of the 2014–2015 influenza season (Figure 1). Antigenic site B of HA clades 3C.3a and 3C.2a viruses differ (Table 1), and future studies will need to be completed to rigorously define the specificity of Abs elicited by the A/Switzerland/9715293/2013 vaccine strain.

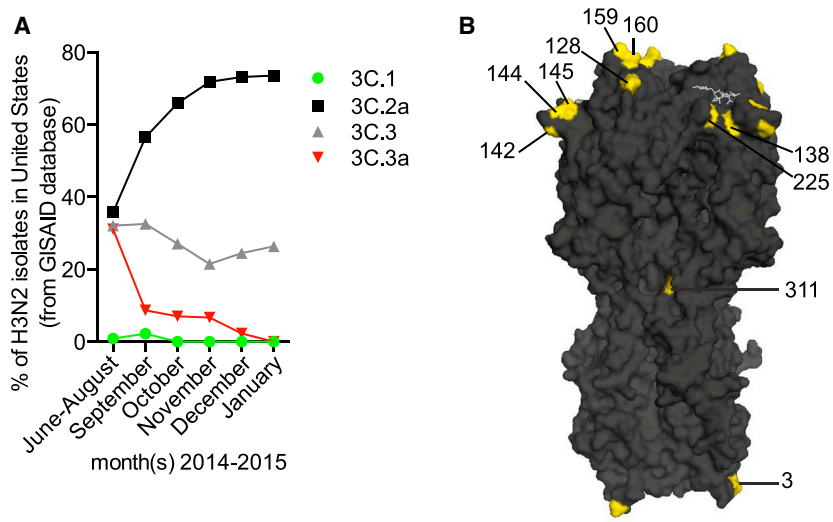

Figure 1. Genetically Distinct H3N2 Viruses Circulated during 2014–2015 Influenza Season in the United States

(A) HA sequences deposited on the GISAID database were analyzed. All sequences deposited by United States laboratories were included in the analysis. Shown are the percentage of viruses that belong to each HA clade (as defined in the text).

(B) The H3 structure (Protein Data Bank: 1HGG) is shown with residues from Table 1 shown in yellow.

(C and D) ELISAs were completed with plates coated with VLPs containing A/Texas/50/2012-WT HA (A/TX/12-WT), A/Texas/50/2012-F159S HA (A/TX/12-F159S), or A/Port Chalmers/1/1973 HA (A/PC/73). ELISAs were completed with A/Texas/50/2012 ferret anti-sera (C) or the F49 mAb (D) that binds to a conserved region of the HA stalk. Shown are mean and SEM of triplicate samples. Data are representative of two independent experiments.

Anti-sera Isolated from Most Humans Vaccinated with A/Texas/50/2012 Possess Abs against HA Antigenic Site B

Next, we completed HAI assays using human sera collected pre- and post-vaccination with the 2014–2015 seasonal influenza vaccine, which contains the A/Texas/50/2012-like H3N2 strain. We completed HAI assays using the A/Texas/50/2012 strain and the A/Texas/50/2012 strain engineered to have the F159S HA mutation. The A/Texas/50/2012 strain has been in seasonal influenza vaccines since the 2013–2014 season and many individuals in our study had high A/Texas/50/2012 Ab titers that were focused against antigenic site B prior to immunization (Table 3). Following vaccination, most individuals either mounted or maintained Abs that had reduced reactivity to viruses possessing the F159S HA mutation (Table 3). Importantly, we were able to detect antigenic site B-specific Ab responses in humans that represent a fairly large age range (21–50 years old). It is interesting that some humans did not mount Ab responses that were specific for antigenic site B following vaccination. Our previous studies suggest that influenza pre-exposures can alter the immunodominance of H1N1 Ab responses (Li et al., 2013b; Linderman et al., 2014), and it is possible that individuals who do not mount site B-specific Ab responses have unique H3N2 pre-exposure histories.

Anti-A/Texas/50/2012 Antigenic Site B Abs Are Neutralizing

Next, we tested whether anti-A/Texas/50/2012 antigenic site B Abs identified in ferrets and humans are neutralizing. We completed in vitro neutralization assays with A/Texas/50/2012 and A/Texas/50/2012-F159S using sera samples isolated from humans 21 days post-vaccination. We focused on samples that had the largest HAI differences using A/Texas/50/2012 and A/Texas/50/2012-F159S viruses (Table 3). We also completed in vitro neutralization assays with antisera isolated from ferrets recovering from infection with the A/Texas/50/2012 strain. Our in vitro neutralization results mirrored our HAI results (Table S2). All seven human sera samples and the ferret sera sample tested had dramatically decreased in vitro neutralization titers using the A/Texas/50/2012-F159S virus compared to the A/Texas/50/2012-WT virus (Table S2).

DISCUSSION

There was a clear H3N2 vaccine mismatch during the 2014–2015 influenza season (Broberg et al., 2015; D'Mello et al., 2015; Flannery et al., 2015; Pebody et al., 2015), and it is important to identify specific HA mutations that have lead to this mismatch.

Table 2. Analyses of Ferret and Sheep Anti-sera Raised against the A/Texas/50/2012 and the A/Switzerland/9715293/13 Strains

	Sera		
Viruses	Ferret α-A/Texas/50/12	Sheep α-A/Texas/50/12	Sheep α-A/Switzerland/9715293/13
A/Texas/50/12-WT	960	10,240	2,560
A/Texas/50/12-N128A	1,280	10,240	2,560
A/Texas/50/12-A138S	480	5,120	1,280
A/Texas/50/12-R142G	640	7,680	1,920
A/Texas/50/12-N144S+N145S	1,280	10,240	3,840
A/Texas/50/12-N145S	480	5,120	1,920
A/Texas/50/12-F159S	240	3,840	3,840
A/Texas/50/12-N225D	640	7,680	2,560
A/Switzerland/9715293/13	60	1,280	2,560

HAI assays were completed using antisera isolated from ferrets 19 days post-infection or sheep 28 days post-infection. Data are representative of three independent assays.

This information is crucial for properly selecting viral strains to be used in future vaccine formulations. In this report, we demonstrate that sheep, ferrets, and humans exposed to the 2014–2015 A/Texas/50/2012 H3N2 vaccine strain mount Ab responses that are targeted against HA antigenic site B. The majority of H3N2 viruses circulating during the 2014–2015 season possessed mutations in antigenic site B (Figure 1A).

Our previous studies indicate that the specificity of human H1N1 Ab responses is shaped by prior H1N1 exposures (Li et al., 2013b; Linderman et al., 2014). We found that most human H1N1 Ab responses are narrowly focused on epitopes that were present in viral strains that circulated during each individual's childhood. It is unclear whether prior H3N2 exposures influence the development of Ab responses to drifted H3N2 strains in a similar manner. Our data indicate that most 21- to 50-year-olds mount anti-A/Texas/50/2012 Abs against antigenic site B. It is important to note that some individuals in our study (four of 32) mounted anti-A/Texas/50/2012 Ab responses that were not directed against antigenic site B (sera from these four individuals had <2-fold change in HAI titer using the A/Texas/50/2012-WT and A/Texas/50/2012-F159S strains). Current studies are under way to investigate whether these four individuals have evidence of unique H3N2 exposures.

The World Health Organization recently recommended that the H3N2 component of the 2015–2016 seasonal vaccine should be updated to include the A/Switzerland/9715293/2013 strain (Anonymous, 2015). We note that this clade 3C.3a virus differs in HA antigenic site B compared to clade 3C.2a viruses, which predominated toward the end of the 2014–2015 Northern Hemisphere influenza season (Figure 1). Clade 3C.2a viruses possess a new predicted glycosylation site in antigenic site B due to S159Y and K160T differences compared to A/Switzerland/9715293/2013. The addition of a new glycosylation site on top of the HA antigenic site B could potentially alter antigenicity and Ab access to this region of HA. In our studies, sera isolated from A/Switzerland/9715293/2013-infected animals reacted equally to the A/Switzerland/9715293/2013 strain and our A/Texas/50/2012 mutant panel; however, additional studies need to be completed to precisely define the specificity of Abs elicited by A/Switzerland/9715293/2013 exposure.

Taken together, our data suggest that mutations in antigenic site B of 2014–2015 H3N2 strains have led to a major antigenic change. This antigenic change is likely responsible for the low vaccine efficacy during the 2014–2015 season. Our studies support the World Health Organization's decision to update the H3N2 component of the 2015–2016 influenza vaccine.

EXPERIMENTAL PROCEDURES

HA Sequences

We obtained HA sequence data from the GISAID website http://platform.gisaid.org/epi3/. Information related to the viral isolates used for these studies is reported in Table S3.

Viruses

We obtained the A/Switzerland/9715293/2013 strain from the National Institute for Biological Standards and Control (NIBSC) in Hertfordshire, UK. We created reverse-genetics-derived viruses that possessed the A/Texas/50/2012 HA. Since we did not have the A/Texas/50/2012 strain when we initiated these experiments, we extracted RNA from the A/Victoria/361/2011 strain and cloned the HA of this virus into the pHW2000 reverse genetics plasmid. We then used QuickChange site-directed mutagenesis kits (Stratagene) to convert the A/Victoria/361/2011HA sequence to the A/Texas/50/2012 HA sequence (by adding T128N, G186V, S198P, S219F, N278K mutations). We then introduced additional mutations from Table S1 into this A/Texas/50/2012 HA sequence. We rescued viruses that possessed the different mutated A/Texas/50/2012 HAs after transfecting 293T/MDCK cell co-cultures with the different HA plasmids and plasmids derived from A/Puerto Rico/8/1934 that encoded for the rest of the influenza genome. All viruses used for antigenic analyses were propagated in 10-day-old fertilized chicken eggs. We used Sanger sequencing to verify that additional mutations did not arise during viral propagation.

Antisera

All sheep anti-sera used in this study were obtained from the National Institute for Biological Standards and Control (NIBSC) in Hertfordshire, UK. Antisera were collected from sheep 28 days post-infection. Ferret antisera used in this study were obtained from the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, CDC, Atlanta, GA. Antisera were collected from ferrets 19 days post-infection. Sera were also collected from humans prior to vaccination and 21 days following vaccination with the 2014–2015 seasonal influenza vaccine. All studies involving the collection and analysis of human sera were approved by the institutional review boards of the Wistar Institute and Vaccine and Gene Therapy Institute of Florida. All

Table 3. Analyses of Sera Isolated from Humans Pre- and Post-vaccination with the 2014–2015 Seasonal Influenza Vaccine that Includes the A/Texas/50/2012 Vaccine Strain

		Pre-vaccination		Post-vaccination	
Sample ID	Age (years)	A/Texas/50/2012	A/Texas/50/2012-F159S	A/Texas/50/2012	A/Texas/50/2012-F159S
01	21	240	40	160	40
02	23	640	240	320	160
03	24	240	80	240	80
04	25	240	80	240	80
05	26	80	<40	640	320
06	27	480	<40	320	<40
07	29	60	<40	120	40
08	30	60	40	80	40
09	30	<40	<40	640	320
10	30	320	240	640	480
11	31	320	80	320	120
12	31	40	<40	60	<40
13	31	<40	<40	40	<40
14	31	<40	<40	160	<40
15	31	40	<40	40	<40
16	34	480	60	640	80
17	35	480	80	320	120
18	35	160	80	160	160
19	35	320	240	640	320
20	35	160	<40	240	<40
21	36	60	<40	120	<40
22	38	<40	<40	120	<40
23	38	<40	<40	240	<40
24	39	240	80	320	80
25	41	320	240	320	240
26	44	<40	<40	80	40
27	44	40	<40	80	80
28	46	<40	<40	80	40
29	48	80	<40	160	40
30	48	<40	<40	60	<40
31	48	<40	<40	80	<40
32	50	80	40	160	80

Sera were collected from humans pre- and post-vaccination with the 2014–2015 seasonal influenza vaccine. HAI assays were completed using A/Texas/50/2012 and A/Texas/50/2012 with the F159S HA mutation. Data are representative of three independent assays.

sera were treated with receptor-destroying enzyme (RDE) for 3 hr prior to antigenic testing.

HAI Assays

HAI titrations were performed in 96-well round bottom plates. Sera were serially diluted 2-fold and added to four agglutinating doses of virus in a total volume of 100 μl. Next, 12.5 μl of a 2% (v/v) turkey red blood cell solution was added. Agglutination was read out after incubating for 60 min at room temperature. HAI titers were recorded as the inverse of the highest dilution that inhibited hemagglutination of turkey red blood cells. Similar results were obtained using guinea pig red blood cells.

ELISA Assays

VLPs expressing A/Texas/50/2012-WT HA, A/Texas/50/2012-F159S HA, or A/Port Chalmers/1/1973 HA were created. Codon-optimized sequences were cloned into the pCMV-Sport6 plasmid. VLPs were rescued by transfecting 293T cells with plasmids expressing HIV gag, A/Puerto Rico/8/1934 NA, HAT (human airway trypsin-like protease), and each HA. VLPs isolated from culture supernatants were concentrated using a 20% sucrose cushion and resuspended in PBS. VLP amounts were normalized in ELISAs using the F49 mAb (Clontech) that binds to a conserved region of the H3 stalk. Goat anti-ferret immunogloublin G (IgG) conjugated to horseradish peroxidase (Abcam) was used to detect binding of A/Texas/50/2012 ferret antisera and goat anti-mouse IgG conjugated to horseradish peroxidase (MP Biomedicals) was used to detect the murine F49 mAb.

Neutralization Assays

In vitro neutralization assays were performed in 96-well flat bottom plates. Sera were serially diluted and then added to 100 TCID50 units of A/Texas/50/2012-WT or A/Texas/50/2012-F159S virus and incubated at room temperature for 30 min. The virus-sera mixtures were then incubated with MDCK cells for 1 hr at 37C. Next, cells were washed, and then serum-free media with TPCK-treated trypsin was added. We visually determined cytopathic endpoints 3 days later. Data are expressed as the inverse of the highest dilution that caused neutralization.

Receptor Binding Assays

As previously described (Li et al., 2013a), turkey red blood cells were pretreated with different amounts of RDE (a neuraminidase) for 1 hr at 37°C. The red blood cells were washed with PBS and added (as 2% v/v solutions) to four agglutinating doses of each virus (as determined using non-treated red blood cells). After a 1-hr incubation, agglutination was measured. Viruses with higher receptor binding avidities are able to bind to red blood cells that are treated with high amounts of RDE (Li et al., 2013a).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.005.

AUTHOR CONTRIBUTIONS

B.S.C. created viruses by reverse genetics, sequenced viruses, completed HAI and ELISA assays, designed experiments, and helped write the manuscript. KP completed in vitro neutralization assays. T.M.R. collected and provided human sera samples. K.A. helped design experiments and edited the manuscript. S.E.H. designed experiments, supervised all experiments, and wrote the manuscript.

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