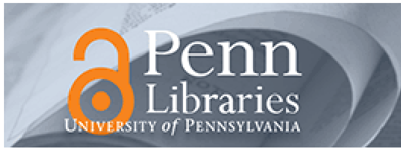

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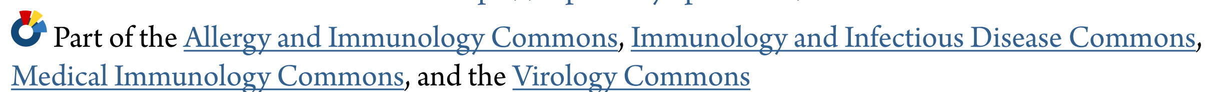
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Vaccine Strain Selection For Influenza A Viruses Is Complicated By Unique Pre-Exposure Histories And Rapid Mutation Of Glycoproteins

Abstract

Influenza viruses cause millions of infections worldwide each year. Influenza viruses constantly acquire mutations in their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), through a process called antigenic drift. HA is the main target of anti-influenza neutralizing antibodies (Abs). Due to antigenic drift, the seasonal influenza vaccines must be updated annually to include the most antigenically relevant strains. Each year, the World Health Organization collects thousands of clinical influenza isolates, propagates them in cell culture, and performs both sequencing and serological analyses to assess the antigenic characteristics of circulating viral strains. In this dissertation, we investigate multiple factors associated with surveillance and vaccine strain selection that could be improved to produce more reliable and effective seasonal influenza vaccines. We first demonstrate that recent H3N2 subtype viral isolates rapidly acquire mutations in both HA and NA when propagated in cell culture, resulting in increased receptor binding avidity or NA-dependent receptor binding, respectively. These mutations impact antigenic analyses that are routinely used for viral surveillance. We then explore how a single mutation in HA antigenic site B contributed to the antigenic drift and subsequent vaccine mismatch of newly emerged H3N2 viruses during the 2014-2015 influenza season. Finally, we found that antisera collected from previously naïve ferrets infected for the first time with influenza (that are commonly used for antigenic analyses during vaccine selection) do not accurately represent the Ab repertoires found in humans that have been infected or vaccinated multiple times with different influenza virus strains. We identified some individuals who have an Ab response targeted to a region of the HA of H1N1 viruses that recently acquired a mutation. Overall, our studies identify ways to improve the process of choosing seasonal influenza virus vaccine strains. We propose that the implementation of "sequence-first" surveillance, new cell culture systems, and the use of clinical human antisera for antigenic characterization of viruses will improve the process of selecting seasonal influenza vaccine strains.

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VACCINE STRAIN SELECTION FOR INFLUENZA A VIRUSES IS COMPLICATED BY UNIQUE PRE-EXPOSURE HISTORIES AND RAPID MUTATION OF GLYCOPROTEINS

Benjamin S. Chambers

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VACCINE STRAIN SELECTION FOR INFLUENZA A VIRUSES IS COMPLICATED BY

UNIQUE PRE-EXPOSURE HISTORIES AND RAPID MUTATION OF GLYCOPROTEINS

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ABSTRACT

VACCINE STRAIN SELECTION FOR INFLUENZA A VIRUSES IS COMPLICATED BY UNIQUE PRE-EXPOSURE HISTORIES AND RAPID MUTATION OF GLYCOPROTEINS

Benjamin S. Chambers

Scott E. Hensley

Influenza viruses cause millions of infections worldwide each year. Influenza viruses constantly acquire mutations in their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), through a process called antigenic drift. HA is the main target of anti-influenza neutralizing antibodies (Abs). Due to antigenic drift, the seasonal influenza vaccines must be updated annually to include the most antigenically relevant strains. Each year, the World Health Organization collects thousands of clinical influenza isolates, propagates them in cell culture, and performs both sequencing and serological analyses to assess the antigenic characteristics of circulating viral strains. In this dissertation, we investigate multiple factors associated with surveillance and vaccine strain selection that could be improved to produce more reliable and effective seasonal influenza vaccines. We first demonstrate that recent H3N2 subtype viral isolates rapidly acquire mutations in both HA and NA when propagated in cell culture, resulting in increased receptor binding avidity or NA-dependent receptor binding, respectively. These mutations impact antigenic analyses that are routinely used for viral surveillance. We then explore how a single mutation in HA antigenic site B contributed to the antigenic drift and subsequent vaccine mismatch of newly emerged H3N2 viruses during the 2014-2015 influenza season. Finally, we find that antisera collected from previously naïve ferrets infected for the first time with influenza (that are commonly used for antigenic analyses during vaccine selection) do not accurately represent the Ab repertoires found in humans that have been infected or vaccinated multiple times with different influenza virus strains. We identified some individuals who have an Ab response

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigenic
ACIP	Advisory Committee on Immunization Practices
ADCC	Antibody dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
BCR	B-cell receptor
BPL	Beta-propiolactone
Cal X-179A	A/California/07/2009 X-179A vaccine strain
CDC	Centers for Disease Control and Prevention
CHOP	Children’s Hospital of Philadelphia
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GISAID	Global Initiative on Sharing All Influenza Data
HA	Hemagglutinin
HAI	Hemagglutination inhibition assay
HAU	Hemagglutination unit
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV	Human immunodeficiency virus
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
Ig	Immunoglobulin
ISG	Inteferon-stimulated gene
JTT	Jones, Taylor, and Thornton replacement matrix
LAIV	Live attenuated influenza vaccine
LRR	Leucine rich repeat
mAb	Monoclonal antibody
MDCK	Madin-Darby canine kidney cell
MDCK-S	Madin-Darby canine kidney cells transfected with 2,6-sialyltransferase
MEM	Minimum essential medium
MHC	Major histocompatibility complex
M1	Matrix protein 1 of influenza virus
M2	Matrix protein 2 of influenza virus
NA	Neuraminidase
NEP	Nuclear export protein of influenza virus
NIBSC	National Institute for Biological Standards and Control
NIH	National Institutes of Health
NLRP3	NOD-, LRR-, and pyrin domain-containing 3
NOD	Nucleotide oligomerization domain
NP	Nucleoprotein protein of influenza virus
NS1	Nonstructural protein 1 of influenza virus
OAS	Original antigenic sin

OAS	2'-5' oligoadenylate synthetase
Oselt	Oseltamivir
PA	Polymerase acidic protein of influenza virus
PBS	Phosphate-buffered saline
PB1	Polymerase basic protein 1 of influenza virus
PB2	Polymerase basic protein 2 of influenza virus
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PFU	Plaque forming unit
pH1N1	Pandemic H1N1 influenza subtype that emerged in 2009
PNGase	Peptide:N-glycosidase
PR8	A/Puerto Rico/08/1934 (H1N1)
QIV	Quadrivalent inactivated vaccine
RBC	Red blood cell
RDE	Receptor-destroying enzyme
RIG-I	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RNaseL	Ribonuclease L
RT-PCR	Reverse transcription polymerase chain reaction
S.A. X-243	A/South Africa/3626/2013 X-243 vaccine strain
SEM	Standard error of the mean
sH1N1	Seasonal H1N1 influenza subtype (circulated 1977-2008)
ssRNA	Single-stranded ribonucleic acid
$TCID_{50}$	50% tissue culture infectious dose
TIV	Tetravalent inactivated vaccine
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidene
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
TRIM25	Tripartite motif containing 25
VGTI	Vaccine and Gene Therapy Institute of Florida
VLP	Virus-like particle
vRNP	Viral ribonucleoprotein
WHO	World Health Organization
WT	Wild type
YOB	Year of birth

CHAPTER 1: INTRODUCTION

Influenza Virus: The Basics

Influenza virus was first isolated and characterized by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw at the National Institute for Medical Research in London in 1933 (Smith et al., 1933). Since that day, much research has been carried out to better understand the importance of influenza virus in the human population. A member of the Orthomyxoviridae family, three main genera of influenza viruses have been discovered, isolated, and characterized. Influenza A viruses, which my work focuses on, circulate most widely and cause the most severe disease in humans (Taubenberger and Morens, 2008). Influenza B viruses only infect mammals and cause less severe infections (Guan et al., 2011). And a lineage of influenza C viruses that mostly infects pigs has also been discovered (Guo et al., 1983). The natural reservoir of influenza A virus is aquatic birds or waterfowl, such as ducks and geese (Hinshaw et al., 1980). However, it is able to infect a wide array of species including pigs, horses, whales, bats, dogs, and of course, humans (Webster et al., 1992).

Influenza viruses cause millions of infections and thousands of deaths in the United States each year (Kostova et al., 2013; Reed et al., 2015). During 2013 alone, influenza infections resulted in an estimated 57,000 deaths in the United States (Xu et al., 2016). The secret behind influenza's ability to remain such a large public health threat on an annual basis is its ability to adapt and mutate to escape detection by prior immunity in the population. Influenza viruses possess a negative-sense single-stranded RNA genome with eight separate gene segments that encode at least 15 proteins (Muramoto et al.,

2013). The viral capsid consists of matrix protein 1 (M1) and matrix protein 2 (M2) (Lamb et al., 1981). There are two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), present on the surface of the virion that are used for attachment and release, respectively (Gamblin and Skehel, 2010). Nonstuctural protein 1 (NS1) is an immune system antagonist with multiple functions (Fernandez-Sesma et al., 2006), and nuclear export protein (NEP) is used primarily for nuclear export of replication products (O'Neill et al., 1998). Within the virion, influenza carries its own RNA-dependent RNA polymerase that consists of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). The polymerase forms a complex with the viral RNA genome and nucleoprotein (NP), called the viral ribonucleoprotein (vRNP) particle, which is essential for replication (Biswas et al., 1998). The RNA-dependent RNA polymerase encoded by influenza lacks proofreading and introduces an average of one mutation in each replicated genome (Drake, 1993). The introduction of point mutations by the polymerase and the rearrangement of gene segments between different influenza strains leads to the high adaptive ability of influenza viruses.

The Immune Response Against Influenza A Virus

During a human infection, influenza virus mainly replicates within the epithelial cells of the host’s upper respiratory tract. The first line of defense for the host is the innate immune response. Like in many other viral infections, the innate immune response is key in fighting off severe influenza infections. Influenza virus is recognized by multiple pattern recognition receptors: Toll-like receptors (TLRs) recognize dsRNA and ssRNA (Finberg et al., 2007), retinoic acid-inducible gene I (RIG-I) recognizes the 5’-

triphosphate of viral RNA (Kato et al., 2006), and NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) detects cellular damage due to influenza replication (Allen et al., 2009). Type I interferons (IFNs) are produced and IFN-stimulated genes (ISGs) are activated to limit influenza replication. A few of the key ISGs include MX proteins, IFN-inducible transmembrane (IFITM) proteins, and ribonuclease L (RNase L). MX proteins expressed in humans have potent anti-influenza virus activity by blocking assembly of functional vRNPs (Verhelst et al., 2012). IFITM3 is a transmembrane host protein that limits influenza entry into the cytosol and IFITM3-deficient mice are highly susceptible to influenza infection (Bailey et al., 2012; Feeley et al., 2011). RNase L works in conjunction with 2'-5'-oligoadenylate synthase (OAS) to bind and degrade viral and cellular ssRNAs (Wreschner et al., 1981), and RNaseL-deficient mice are more susceptible to influenza infection (Zhou et al., 2013). The innate immune response is so effective that the influenza virus encodes its own protein, NS1, which is responsible for antagonizing and inhibiting the innate immune response. NS1 has been shown to bind and sequester dsRNA away from OAS so that RNase L remains inactive (Min and Krug, 2006). NS1 also interacts with TRIM25 to inhibit ubiquitination of RIG-I, and thus prevent RIG-I signal transduction (Gack et al., 2009). NS1 plays multiple other roles in evading host cell detection and limiting the innate immune response.

Early during infection, professional antigen-presenting cells (APCs), such as dendritic cells and macrophages, take up and process influenza virus antigen. This antigen can come from whole virus, from replication intermediates, or from influenza-infected cells taken up by phagocytosis (Mintern and Villadangos, 2015). The influenza antigens are then presented on the surface of the APCs in association with major-

histocompatibility complexes (MHCs) I or II, to activate both $CD8^+$ and $CD4^+$ T cells (Alam and Sant, 2011; Turner et al., 2013). Mature B cells expressing a B cell receptor (BCR) on their surface are also activated by recognizing viral antigen, and experience proliferation through $CD4^+$ T cell-mediated help in the germinal centers (Victora and Nussenzweig, 2012). B cells activated early after infection produce IgM antibodies (Abs). Activated B cells then undergo class-switching and begin producing IgG Abs that are capable of neutralizing influenza virus in multiple ways, including binding to HA and blocking receptor binding, inhibiting membrane fusion, or initiating Ab-dependent cell-mediated cytotoxicity (ADCC) (Jegaskanda et al., 2013). Some B cells in the mucosal tissues produce high levels of IgA instead, in order to prevent further infection of mucosal surfaces, especially in the nasal passage (Renegar et al., 2004). Two to four weeks after primary infection, Ab production is at its peak (Clements and Murphy, 1986). During the primary response, a long-lived memory B cell pool is established (Buchner et al., 1977; Skountzou et al., 2014). This memory B cell pool produces Abs specific for the influenza strain causing the primary infection, and these cells can be recalled following subsequent influenza virus exposures with antigenically similar strains.

Following a secondary infection with influenza, the memory B cell pool provides a rapid production of influenza-specific Abs that reach their peak production much earlier than in the primary response. However, in order to be reactivated, the memory B cell must be specific for an epitope that is conserved between the previous infecting strain and the current infecting strain. Once triggered, the B cell proliferates and undergoes somatic hypermutation in order to generate Abs with higher affinity and increased neutralization against the influenza antigen (Eisen, 2014). Once again, a new pool of memory B cells is

established during this secondary response. Throughout a lifetime, humans are infected and/or vaccinated multiple times with antigenically distinct influenza viruses. During each subsequent exposure, a memory B cell response is recalled and further shaped through somatic hypermutation.

Genetic Reassortment and Antigenic Shift

The main targets of anti-influenza neutralizing Abs are the two surface glycoproteins, HA and NA. One mechanism employed by influenza viruses to escape B cell memory and the anti-HA/NA Ab response is to swap gene segments with other strains of influenza virus. Gene segments can be swapped between two different influenza virus strains during co-infection of the same cell. This process is called reassortment, and different combinations of the eight influenza gene segments can result in the emergence of new influenza subtypes or new influenza strains with altered host specificity and increased virulence (Steel and Lowen, 2014). The subtype of each virus is defined by the antigenic properties of their HA and NA glycoproteins. Currently, there are 18 known HA subtypes and 11 known NA subtypes (Tong et al., 2013). Over the past century, several different subtypes of influenza A virus have circulated in the human population (Fig. 1). Reassortment that results in the emergence of a new viral subtype with antigenically novel HAs and NAs is called antigenic shift (Nelson and Holmes, 2007).

Antigenic shift occurs every time a new pandemic emerges in the human population (Fig. 1). The pandemic of 1918 that claimed an estimated 50 million lives has been attributed to the emergence of an H1N1 influenza A virus (Johnson and Mueller,

2002; Taubenberger et al., 1997). These H1N1 viruses continued circulating in the human population until 1957, when a new subtype (H2N2) emerged following the reassortment of the circulating human H1N1 viruses with the PB1, HA, and NA genes of an avian H2N2 virus (Schafer et al., 1993). The "Asian Flu" of 1957 was a milder pandemic but still claimed 70,000 lives in the United States (Noble, 1982). Another antigenic shift event occurred in 1968 when the H3N2 "Hong Kong Flu" pandemic emerged and killed 34,000 people in the United States (Noble, 1982). In this case, the prior H2N2 viruses acquired the HA and PB1 genes from an avian H3N2 virus (Gething et al., 1980). Antigenically drifted H3N2 viruses from this pandemic are still circulating today. In 1977, H1N1 viruses returned to circulation, likely following a lab containment accident that reintroduced H1N1 viruses into a partially naïve population (Kendal et al., 1978; Webster et al., 1992). These H1N1 viruses were genetically similar to those that had circulated in the early 1950s (Nakajima et al., 1978; Scholtissek et al., 1978). Older adults had prior immunity from exposure to similar strains, and therefore the majority of disease burden occurred in children and young adults under the age of 25 (Kilbourne, 2006). These H1N1 viruses are now referred to as the seasonal H1N1 (sH1N1) viruses as a way to separate them from the antigenically distinct pandemic (pH1N1) viruses that emerged in 2009.

The "Swine Flu" of 2009 resulted in many severe infections, surprisingly only 5% of them being in adults over the age of 51 (Dawood et al., 2009). Elderly individuals were thought to be protected due to similarities in the HA between the pH1N1 viruses of 2009 and the H1N1 viruses that circulated prior to the 1950s (Skountzou et al., 2010). As discussed earlier in this chapter, memory B cells primed early in life can be recalled when

confronted with new strains that possess B cell epitopes conserved in older viral strains. The pH1N1 viruses were the result of a quadruple reassortment between gene segments from human H3N2 viruses, classical swine viruses, Eurasian swine viruses, and a North American avian virus introduced through a swine lineage (Garten et al., 2009). Approximately 20-30% of the amino acid residues in HA are different between pH1N1 viruses and sH1N1 viruses (Li et al., 2012). The sH1N1 viruses quickly became extinct following the emergence of pH1N1 viruses, possibly because pH1N1 viruses had higher fitness (Perez et al., 2009) or because pH1N1 viruses elicited cross-reactive stalk Abs and anti-NA Abs that bound with high affinity and completely neutralized the sH1N1 viruses (Palese and Wang, 2011). Currently, both pH1N1 and H3N2 influenza A viruses are actively circulating in the human population on an annual basis and both will be discussed further in this dissertation.

Antigenic Importance of Hemagglutinin and Antigenic Drift

As stated previously, there are two glycoproteins exposed on the surface of influenza viruses. The surface is covered in a 10:1 ratio of HA to NA (Mitnaul et al., 1996). NA is a tetramer that is mainly responsible for the release of newly formed virions from the surface of host cells. HA exists as a trimer on the virion surface and is responsible for binding to the target cell via its receptor, a terminal sialic acid residue on the surface of the host cell. HA is synthesized as a precursor, called HA0, which must be cleaved by a trypsin-like protease to form the activated confirmation of HA1 and HA2 (Gething et al., 1986). After binding to the sialic acid receptor through HA1, the virus is taken into the cell through dynamin-dependent, clathrin-mediated endocytosis (Patterson

et al., 1979). A decrease in pH within the endosome triggers a conformational change that exposes the fusion peptide within HA2 (Skehel et al., 1982). The fusion peptide then imbeds into the host endosomal membrane and forms a pore for release of the vRNPs into the cytoplasm (Stegmann et al., 1991). Abs that bind to HA can neutralize the virus in multiple ways, including prevention of HA0 cleavage, obstruction of receptor binding, and inhibition of membrane fusion (Brandenburg et al., 2013).

Previous work completed in the 1970s and 1980s mapped the immunodominant epitopes on HA where most anti-influenza neutralizing Abs bind. These immunodominant epitopes are referred to as antigenic sites. Escape mutants were generated in the presence of monoclonal Abs to identify the four antigenic sites (Sa, Sb, Ca, and Cb) of H1N1 viruses (Caton et al., 1982). Sequence data and the interpretation of past antigenic drift mutations were studied to identify the five antigenic sites (A, B, C, D, and E) of H3N2 viruses (Wiley et al., 1981). The antigenic sites are located across the head of the HA trimer (Fig. 2). This region is the most exposed and thus the most accessible for binding of Abs. Neutralizing Abs that bind the more conserved stalk domain located closer to the membrane have also been identified (Corti et al., 2011; Ekiert et al., 2009; Okuno et al., 1993; Sangster et al., 2013; Sui et al., 2009), but the vast majority of anti-influenza neutralizing Abs bind to the easily accessible globular head domain of HA. It is also important to note that not all neutralizing Abs bind to one of these classic antigenic sites. The classic antigenic sites can be thought of as defining a general location on the HA trimer, rather than identifying a very specific set of individual residues that are involved in Ab binding.

Because most neutralizing Abs bind to these antigenic sites, they are also the most common sites for the accumulation of antigenic drift mutations. Genetic drift is defined as the continuous accumulation of nonsynonymous mutations in viral gene segments; a result of the RNA-dependent RNA polymerase that lacks proofreading (Nelson and Holmes, 2007). Antigenic drift occurs when immune pressure leads to the fixation of those mutations that abrogate the binding of neutralizing Abs (Fitch et al., 1997). Throughout the evolution of H3N2 influenza viruses between 1968 and 2003, over 70 mutations became fixed in the H3N2 HA; 94% of which were in the classic H3N2 antigenic sites identified previously (Smith et al., 2004; Wiley et al., 1981). A recent transposon mutagenesis screen concluded that HA, the globular head domain in particular, is the most permissible region to mutations in the influenza genome (Heaton et al., 2013). Viral mutations can also alter viral fitness through mechanisms that do not involve abrogating Ab binding. For example, mutations throughout the influenza genome can change receptor specificity (Zhang et al., 2013a), alter neuramindase activity (Richard et al., 2008), switch host tropism (Mehle et al., 2012), increase polymerase activity (Liu et al., 2016), and promote nuclear import of vRNP particles (Sediri et al., 2015).

It is important to understand how mutations in HA can lead to changes in host tropism. Influenza viruses attach to cells by binding to sialic acid receptors. Sialic acid is linked to galactose through either an α2,3 or an α2,6 linkage. Most avian influenza viruses are specific for α2,3-linked sialic acids while most human influenza viruses are specific for α2,6-linked sialic acids (Rogers et al., 1983). Mutations in HA can alter sialic acid specificity and allow viruses to jump from one species to the other (Tharakaraman et

al., 2013). This scope of specificity makes sense when considered in the context of which sialic acids coat the cells in certain species. In aquatic birds, their guts (the main site of influenza virus replication in birds) are coated with predominantly α2,3-linked sialic acids (Pillai and Lee, 2010). However, the human upper respiratory tract (where influenza mainly replicates in humans) is mostly coated in α2,6-linked sialic acids (Shinya et al., 2006; Thompson et al., 2006). Interestingly, the human lower respiratory tract does contain α2,3-linked sialic acids, which may help to explain why avian-derived influenza viruses like H5N1 and H7N9 often spread lower into the lungs and cause pneumonia and death in human infections (Knepper et al., 2013; Zeng et al., 2013). The gut of swine contains a mixture of both α2,3- and α2,6-linked sialic acids, explaining why they can act as so-called "mixing vessels" for influenza reassortment of multiple types of strains (Trebbien et al., 2011). Other than switching host tropism, mutations in the HA receptor binding domain can simply increase or decrease receptor binding avidity (Lin et al., 2012). In fact, many mutations in HA that alter antigenicity also increase or decrease receptor binding avidity (Hensley et al., 2009). This leads to complications during antigenic analyses of recently emerged mutant viruses.

Original Antigenic Sin

Over 50 years ago, Thomas Francis, Jr. coined the term "original antigenic sin" as a way to describe the observation that even when infected with a novel influenza strain, humans will produce Abs against previously encountered strains at the expense of mounting effective responses against the current novel strain (Davenport et al., 1953; Francis, 1960). Ever since then, original antigenic sin (OAS) has been explored more in

depth to better establish the role it plays in secondary immune responses against influenza virus. Various studies have focused on antigenic seniority and the role of OAS in H3N2 responses (Lessler et al., 2012), longitudinal analyses of H1N1, H2N2, and H3N2 responses (Miller et al., 2013), and Ab responses against the 2009 pH1N1 virus (Wrammert et al., 2011). OAS following influenza infection has been observed in multiple other animal models including rabbits (Fazekas de St and Webster, 1966b), mice (Virelizier et al., 1974b), and ferrets (Webster, 1966). Apart from other animal models, OAS has also been detected in other pathogens. For example, OAS has been observed following sequential dengue virus infections and may play a role in shaping the Ab response against human immunodeficiency virus (HIV) over time (Ciupe et al., 2011; Halstead et al., 1983).

Mechanisms that lead to OAS are not completely understood, but OAS likely occurs when cross-reactive B cell clones are recalled from the memory pool during infections with antigenically related influenza strains (Li et al., 2013b; Wrammert et al., 2011). The cross-reactive OAS Abs these memory B cells produce quickly eliminate the new antigen before any novel Ab responses can be established (Kim et al., 2009). OAS has been hypothesized to lead to reduced vaccine efficacy. However, recent studies have shown that OAS Abs that bind with low affinity can afford protection against secondary viral challenges (Linderman and Hensley, 2016). Recent studies have also explored how the effects of OAS can be avoided during vaccination. Repeated vaccination with the same novel strain or the use of adjuvants potentially limits OAS (Kim et al., 2012). The impact of OAS on the secondary immune response against influenza virus will be explored in Chapter 4.

Current Vaccination Methods

The best approach for limiting influenza infection in the human population is annual vaccinations with currently circulating influenza strains. Jonas Salk and Thomas Francis, Jr. developed the first influenza vaccine in 1938 by using ultraviolet radiation to inactivate the virus (Salk et al., 1940). Today, the most common approach is still to vaccinate with an inactivated virus; specifically, an inactivated split virus or subunit vaccine that is grown in eggs (Wong and Webby, 2013). These inactivated vaccines contain an H1N1 influenza A virus component, an H3N2 influenza A virus component, and one or two influenza B components. They are called trivalent inactivated vaccines (TIVs) or quadrivalent inactivated vaccines (QIVs) depending on how many influenza B strains they include, and they are injected into the muscle of recipients. Inactivated vaccines have been used for over 70 years and have a good history of reducing disease in the human population (Hannoun, 2013). During a typical season, the inactivated vaccines have an efficacy of 60-70% (Treanor et al., 2012). Over the past couple decades, the recommendation for vaccination in the United States has expanded to include the entire population. Previously, influenza vaccination was focused primarily on at-risk groups including healthcare workers, pregnant women, young children (<6 years old), and the elderly (>65 years old) (Hannoun, 2013). However, as the public health impact and severity of influenza virus has increased and become better understood, the United States began recommending in 2010 that everyone over the age of 6 months be vaccinated against influenza virus on an annual basis (Fiore et al., 2010).

Another current method of influenza vaccination is the use of attenuated viruses. For example, FluMist is a licensed vaccine that contains a whole virus that can only grow

at lower temperatures (25°C). It is injected into the nose of the recipient where it can replicate and elicit an immune response. However, the vaccine strain is unable to expand into the lungs and cause an infection because it cannot replicate at the internal body temperature of 37°C. The live-attenuated influenza vaccines (LAIVs) do present a couple advantages over the inactivated subunit vaccine. First, LAIVs replicate in the nasal cavity and thus elicit T-cell responses that are an important part of influenza immunity (Mohn et al., 2015). Second, LAIVs also elicit robust mucosal IgA Ab responses (Ambrose et al., 2012) compared to the inactivated subunit vaccines which elicit primarily IgG-dominated Ab responses (Cox et al., 1994). Although early uses of the LAIVs were successful, recent formulations of FluMist in particular have elicited very poor responses and have resulted in very low vaccine efficacies. For example, the LAIV vaccine efficacy was reported at only 3% during the 2015-2016 season (Anonymous, 2016). This has led to the recent recommendation by the CDC to only use inactivated subunit vaccines during the upcoming 2016-2017 influenza season (Anonymous, 2016).

The future of influenza vaccination is targeted at generating more cross-reactive vaccine responses, or even a universal vaccine that will elicit lifelong influenza immunity. One approach currently being explored is the use of peptide antigens that contain epitopes commonly recognized by B cell or T cell responses. For example, an NP epitope encased in a liposome has been shown to elicit potent T cell responses and reduce viral lung titers in influenza-infected mice (Ninomiya et al., 2002). A second approach being pursued is recombinant proteins. Headless HA proteins expressed on virus-like particles (VLPs) or on nanoparticles have shown promise in mice and ferrets by increasing the cross-reactivity of the anti-influenza Ab response by targeting it towards

the more conserved stalk domain of HA (Steel et al., 2010; Yassine et al., 2015). Another version of recombinant proteins being employed to elicit stalk-focused Abs is chimeric HA proteins. By sequentially vaccinating with recombinant proteins that all share the same HA stalk but contain different HA globular heads, the recalled Ab response is focused against the conserved stalk domain (Krammer et al., 2014; Krammer and Palese, 2013). These along with many other approaches (such as M2-focused Abs, adjuvants, and DNA-based vaccines) could end up being the future of influenza vaccination. But in the mean time, it is important to continue improving the current method of vaccination, strain selection, and vaccine manufacturing.

Influenza Virus Surveillance and Vaccine Strain Selection

Currently, the influenza vaccine must be updated each year to possess the most relevant strains of H1N1, H3N2, and influenza B viruses. The constant accumulation of mutations in HA via antigenic drift can make vaccine selection a very difficult process. In order to select the best possible vaccine strains on an annual basis, the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) employ constant surveillance (Stöhr et al., 2012). Throughout the year, thousands of influenza isolates are collected from infected individuals. These strains are grown in cell culture or eggs to obtain a workable stock, antigenically characterized, and sequenced (Krauss et al., 2012; Stöhr et al., 2012). When antigenically distinct viruses are detected, the vaccine is updated. The most common method employed for determining antigenic differences compared to the current vaccine strain is the serological assay called the hemagglutination inhibition (HAI) assay (Stöhr et al., 2012). The HAI assay measures the

amount of antisera required to inhibit a virus from binding to its sialic acid receptor. The WHO and CDC mainly use antisera collected from previously naïve ferrets infected for the first time with the current influenza vaccine strain (Stöhr et al., 2012). This "primary ferret antisera" is then used in HAI assays and in *in vitro* neutralization assays to detect when circulating strains escape detection by the Abs elicited by the current vaccine strain. The *in vitro* neutralization assay is another serological assay and it measures the ability of antisera to neutralize the growth of virus in cell culture. It is a more reliable assay in identifying all functionally active Abs that neutralize virus, compared to the HAI assay which can only detect Abs that block virus-sialic acid interactions (Bachmann et al., 1999; Zhu et al., 2011). For example, there are HA stalk-binding Abs that are HAI-negative (do not affect binding) but still neutralize virus by blocking membrane fusion or inhibiting proteolytic cleavage of HA0 (Pica et al., 2012). Therefore, HAI assays and *in vitro* neutralization assays using mainly sera collected from previously naïve ferrets are used to characterize the antigenic properties of newly emerged viruses.

However, multiple factors can complicate the vaccine selection process. For example, one caveat is that the vaccine strains need to be chosen over nine months before the actual influenza season begins in order to manufacture enough vaccine doses. This means that between the time that the vaccine composition is selected and the time it is actually given to humans, the circulating viruses could acquire additional mutations and establish an HA identity that is undetectable by the vaccine-elicited Ab response. Every few years, antigenic drift that occurs leading up to an influenza season results in a vaccine mismatch where vaccine efficacy is drastically reduced (Carrat and Flahault, 2007; Chambers et al., 2015; Xie et al., 2015). Another complicating factor is that there

are no established definitions for how much antigenic change is required before a vaccine update is called for. The HAI and *in vitro* neutralization assays can quantify the difference in ability of antisera to bind and neutralize different viruses. However, there is no set amount of antigenic change that triggers an automatic vaccine update. The choice to update the vaccine is left up to the consideration and conclusions of the WHO and CDC. In general, the H3N2 and influenza B components of the vaccine have been updated every 2-4 years on average (Fouchier and Smith, 2010; Smith et al., 2004). However, the current H1N1 vaccine strain has not been updated since 2009, when the pH1N1 viruses first emerged. The last major complication is that sera collected from previously naïve ferrets infected with influenza virus a single time are used to represent human immunity. Although ferrets are a great model of influenza infection and immunity, recent studies have challenged whether ferret antisera following a single infection can truly represent the spectrum of Ab response specificities found in the human population (Fonville et al., 2016; Li et al., 2013b).

Ferrets and Mice as Models for Influenza Infection

Two of the most commonly used animal models for influenza virus infection and immunity are mice and ferrets. The effective use of mice (Shope, 1935) and ferrets (Shope, 1934) in influenza research was established immediately after the first isolation of influenza virus in 1933. As mice and ferrets will both be employed in the chapters that follow, it is important to establish both the pros and cons associated with both systems so that data can be accurately interpreted. Mice are a reliable, genetically stable, less expensive, and easily manipulated system used to study influenza virus infections. Mice

become infected when challenged with a sufficient dose of influenza virus and show multiple signs of illness. Weight loss, lethargy, and ruffled fur are all indications of infection severity and have been used as reliable measurements in many early vaccine and antiviral efficacy studies (Gubareva et al., 1998; Tumpey et al., 2002). Passive Ab transfer experiments that measure the ability of Abs to prevent severe infection are also commonly carried out in mice (Francis and Magill, 1935; Renegar and Small, 1991). However, using mice as a model for influenza research does present multiple drawbacks. Influenza strains often acquire mutations and become adapted to replicate well in mice (Keleta et al., 2008). For example, one of the most commonly used influenza lab strains, A/Puerto Rico/08/1934 (PR8), is a heavily mouse-adapted strain (Gerber et al., 1955). Comparisons between mouse-adapted and human-adapted strains cannot always be made with certainty due to adaptive mutations that have arisen in the virus when passaged in mice. A second main concern with mice is that their presentation of illness does not match the human response to an influenza infection. Mice do lose weight, but do not develop a fever during infection (Dybing et al., 2000). Viral replication and tissue damage is concentrated in the lower respiratory tract in mice, instead of the upper respiratory tract like it is in humans (Belser et al., 2010). Transmission studies with H1N1, H3N2, and H5N1 viruses have also proven unsuccessful in mice (Lowen et al., 2006). Overall, mice are a good model for answering some experimental questions but cannot provide a true representation of human influenza infection.

All the limitations of the mouse model actually represent the strengths of the ferret model. Ferrets are easily infected with non-adapted human influenza viruses and pathogenesis presents in very similar ways compared to human infections (Herlocher et

al., 2001). For example, ferrets infected with influenza virus experience respiratory distress, fever, nasal discharge, anorexia, and lethargy similar to humans (Smith et al., 1933). Developing classic clinical symptoms makes ferrets great models for testing antiviral efficacy (Govorkova et al., 2007; von Itzstein et al., 1993). Another major advantage to using ferrets is that they can transmit influenza virus via aerosols. Aerosol transmission is the major method of transmission in human influenza outbreaks. Many successful transmission studies have been conducted using the ferret model, including transmission of H7N2 (Belser et al., 2008), H9N2 (Wan et al., 2008), and the recent swine-origin pH1N1 of 2009 (Munster et al., 2009). The major downside to using the ferret model for influenza research is that they are expensive and require specialized animal facilities (Maher and DeStefano, 2004).

The ferret model is employed as the main animal model for virus surveillance and vaccine selection (Belser et al., 2011). And although recent studies have concluded that sera collected from previously naïve ferrets infected with influenza for the first time cannot represent the entire spectrum of anti-H1N1 Ab specificities found in humans, ferrets sequentially infected with antigenically distinct H1N1 strains do recapitulate the unique Ab responses seen in humans (Li et al., 2013b; Linderman et al., 2014). However, previously naïve ferrets infected with influenza a single time continue to be the main standard used in viral surveillance and vaccine strain selection.

Main Experimental Questions

Surveillance for vaccine selection currently relies on collecting viral isolates, growing them in cell culture and eggs, characterizing their antigenic profile using sera

collected from previously naïve ferrets infected with influenza for the first time, and sequencing their HA gene (Stöhr et al., 2012). However, multiple steps could be taken to improve this process and select more effective influenza vaccine strains in the future. The chapters below will explore the main aspects of influenza surveillance and vaccine strain selection that need to be improved.

First, we will investigate whether antigenicity of recent H3N2 clinical isolates is affected by cell culture propagation. Recent H3N2 influenza viruses have poor receptor binding avidity and grow poorly in cell culture because they cannot bind well to their sialic acid receptors for entry (Gulati et al., 2013; Lin et al., 2012; Nobusawa et al., 2000). We find that during propagation in cell culture, H3N2 clinical isolates rapidly acquire mutations in both HA and NA. These mutations result in increased receptor binding avidity, NA-dependent sialic acid receptor binding, and altered antigenic characteristics.

Second, we will explore how a single mutation in an antigenic site of HA can contribute to a major vaccine mismatch. The H3N2 vaccine component failed during the 2014-2015 influenza season due to a mismatch between the vaccine strain and the viral strains that actually circulated. We determined that mutations that arose in the HA globular head were responsible for the antigenic drift that caused the vaccine mismatch. The main contribution was from a single mutation in HA antigenic site B, a region that has been implicated multiple times in the past for antigenic drift mutations that contributed to the evolution of H3N2 viruses (Koel et al., 2013; Popova et al., 2012).

Third, we will examine whether recent mutations in the pH1N1 virus subtype are being antigenically mischaracterized using sera collected from previously naïve ferrets

infected with influenza for the first time. Humans are infected and/or vaccinated with antigenically distinct influenza viruses multiple times throughout their lives. The Ab responses primed early in their lives are recalled during each subsequent secondary infection with antigenically related strains. We find that childhood sH1N1 infections shape the specificity of Ab responses elicited against pH1N1 viruses. Specifically, the Ab responses elicited by pH1N1 viruses are focused on epitopes conserved between the pH1N1 viruses and the sH1N1 viruses that individuals were likely infected with early in life. In Chapter 4, we will explore whether mutations that arise in these unique Ab binding epitopes are being antigenically mischaracterized by the current antigenic analyses used in surveillance that employ mainly antisera isolated from pH1N1-infected ferrets who do not have any prior H1N1 exposures.

Together, our studies demonstrate the importance of updating current surveillance and vaccine strain selection techniques in order to produce more effective seasonal influenza vaccines. We propagated clinical isolates in cell culture, sequenced HA genes, and conducted antigenic analyses with both ferret and human antisera to identify antigenically important mutations. We conclude that recent H3N2 viruses rapidly acquire mutations in MDCK cell culture, single mutations in HA can cause antigenic drift and subsequent vaccine mismatches, different humans have unique anti-pH1N1 Ab responses due to unique pre-exposure histories, and newly arisen mutations are being antigenically mischaracterized by sera isolated from ferrets recovering from primary infections. Due to all these concerns, multiple steps need to be taken to improve upon the current vaccine strain selection process. The use of “sequence-first” surveillance, new cell culture

systems, and sequentially infected ferret or clinical human antisera in antigenic analyses would likely increase vaccine effectiveness.

FIGURES FOR INTRODUCTION

Figure 1

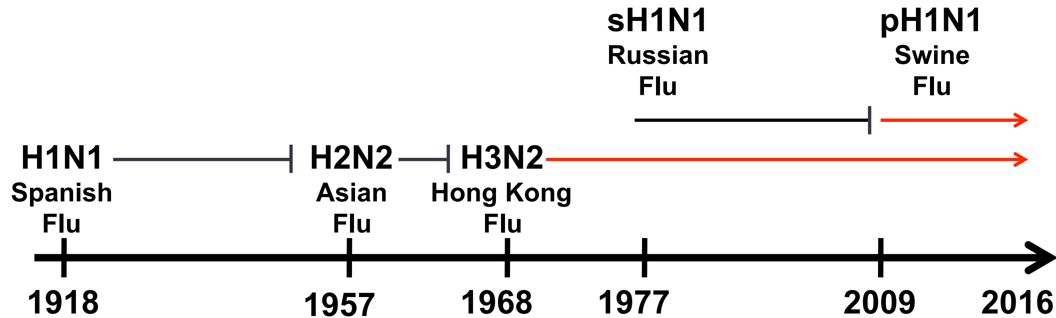

Figure 1. Timeline of influenza A virus circulation in the human population over the past century.

This timeline denotes the circulation of different influenza A virus subtypes in the human population since 1918. The emergence of a new subtype is associated with genetic reassortment and antigenic shift. The continued circulation of a subtype is associated with continuous antigenic drift. The seasonal H1N1 and pandemic H1N1 subtypes are abbreviated as sH1N1 and pH1N1, respectively. The two subtypes currently circulating on a seasonal basis, pH1N1 and H3N2, are highlighted in red.

Figure 2

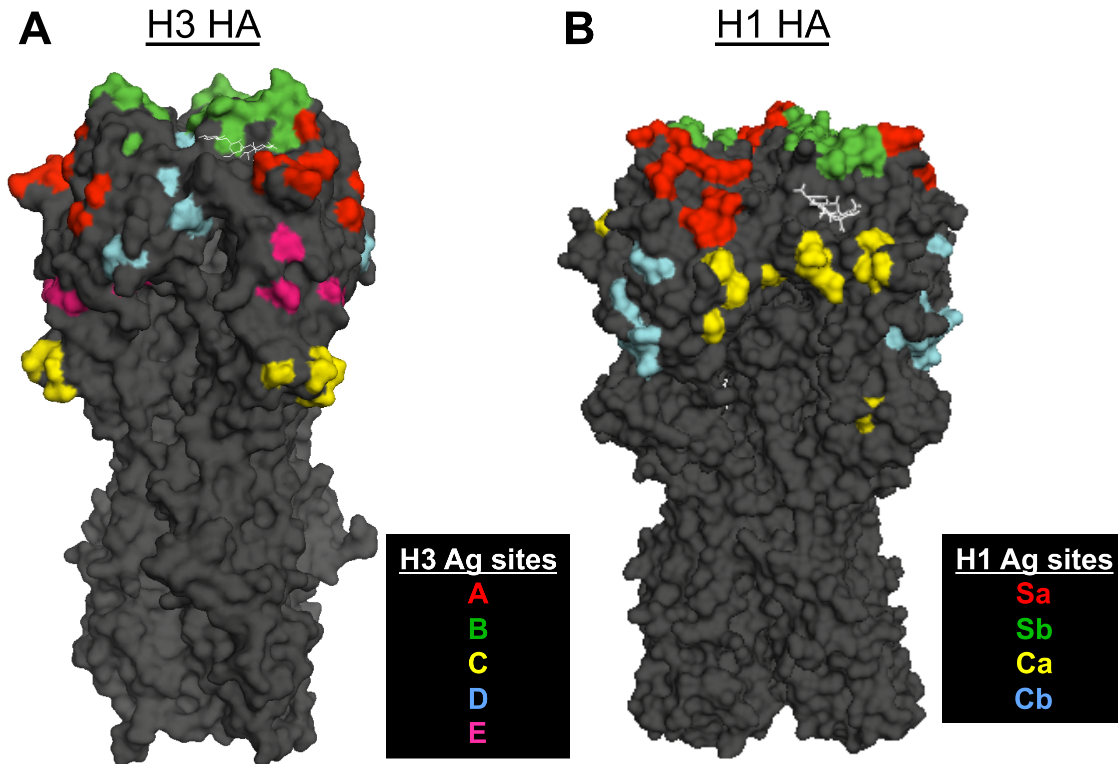

Figure 2. Previously identified antigenic sites mapped on the H3 and H1 crystal structures.

The classic antigenic sites for both H3N2 viruses (Wiley et al., 1981) and H1N1 viruses (Caton et al., 1982) were originally identified in the early 1980s. Here, the five classic antigenic (Ag) sites of H3 HA (A) and the four classic antigenic sites of H1 HA (B) are mapped on the HA trimers (Protein Data Bank (PDB) entries 1HGG and 1RVX) in different colors identified by the legends. The sialic acid receptor is shown in white.

CHAPTER 2: RECENT H3N2 INFLUENZA VIRUS CLINICAL ISOLATES RAPIDLY ACQUIRE HEMAGGLUTININ OR NEURAMINIDASE MUTATIONS WHEN PROPAGATED FOR ANTIGENIC ANALYSES

Parts of this chapter were previously published in:

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SUMMARY

Influenza A viruses continuously acquire mutations in their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), in order to escape neutralizing immune responses and continue circulating. Each year, influenza virus surveillance is required to determine the most antigenically relevant strains to include in the vaccine. Thousands of isolates are collected, expanded in cell culture, and then antigenically characterized and sequenced. Recent H3N2 viruses have drastically decreased receptor binding avidity and thus acquire adaptive mutations when grown in cell culture. We passaged 10 clinical isolates in cell culture and determined that HA and NA mutations are acquired rapidly.

These mutations result in growth to higher infectious titers and increased agglutination of red blood cells. The NA mutations acquired during propagation led to NA-dependent binding of the sialic acid receptor, and the HA mutation led to increased receptor binding avidity. Both of these characteristics have profound effects on antigenic analyses, and could be negatively affecting annual influenza vaccine surveillance.

INTRODUCTION

H3N2 influenza A viruses have circulated continuously in the human population since 1968. In that time, they have steadily acquired mutations in the antigenic sites of both of the glycoproteins present on their surface, hemagglutinin (HA) and neuraminidase (NA) (Yewdell, 2011). This process of acquiring mutations to evade neutralizing antibody (Ab) responses is called antigenic drift and has occurred in H3N2 viruses since 1968 (Smith et al., 2004). HA is the major antigenic target of the humoral immune system, and thus acquires the majority of antigenic drift mutations (Smith et al., 2004). Mutations in HA are able to change both antigenicity and receptor binding avidity simultaneously (Hensley et al., 2009; Underwood et al., 1987). Recent H3N2 viruses have dramatically reduced receptor binding avidity and altered receptor specificity (Gulati et al., 2013; Lin et al., 2012).

Due to antigenic drift, the influenza vaccine must be updated on an annual basis to include the most antigenically relevant strains. Surveillance is conducted continuously to monitor which strains are circulating and when an antigenically drifted strain emerges. Viral isolates are collected from infected individuals, expanded in cell culture to obtain a working stock, sequenced, and then antigenically profiled using serological assays with

sera collected from previously naïve ferrets infected with influenza for the first time (Krauss et al., 2012; Stöhr et al., 2012). The main serological assay used to determine antigenic change is the hemagglutination inhibition (HAI) assay. The HAI assay measures the amount of antisera required to inhibit the agglutination of red blood cells (RBCs) bound by virus (Hirst, 1942). HA is the sole attachment factor for the majority of influenza virus strains, and HA agglutinates RBCs by binding to sialic acid. HAI assays are therefore intended to quantify relative amounts of HA-specific antibodies. However, recent studies demonstrated that some H3N2 and H1N1 strains agglutinate RBCs through NA-sialic acid interactions (Hooper and Bloom, 2013; Lin et al., 2010; Zhu et al., 2012).

With drastically decreased receptor binding avidity, recent H3N2 viruses have difficulty growing in cell culture or eggs and have been shown to acquire mutations in HA and NA (Chen et al., 2010b; Nakowitsch et al., 2014; Nakowitsch et al., 2011; Skowronski et al., 2014; Wang et al., 2013). The extent of these mutations and their effect on antigenic analyses has not yet been investigated in depth. In this study, we completed a series of experiments to determine whether HA and/or NA mutations commonly arise when contemporary clinical H3N2 isolates are expanded in cell culture and the impact they have on antigenic testing. As expected, we observed the emergence of multiple mutations in both HA and NA after a single passage in cell culture. NA mutations resulted in NA-dependent agglutination and an HA mutation resulted in increased receptor binding avidity, both leading to the alteration of antigenic analyses.

RESULTS

Expanded clinical H3N2 isolates bind cells in an NA-dependent manner

During the 2012-2013 influenza season, respiratory secretions were collected at the Children's Hospital of Philadelphia from 10 children (ages 1 month to 7 years old) infected with H3N2 influenza virus. Remaining de-identified clinical samples were passaged two times on Madin-Darby canine kidney (MDCK) cells, which are routinely used by surveillance labs for isolating human viruses (Krauss et al., 2012). After each passage, supernatants were collected and analyzed. Infectious titer was quantified by a 50% tissue culture infective dose ($TCID_{50}$) assay and agglutination was measured using both turkey and guinea pig RBCs.

Following two passages, 9 of 10 MDCK cultures contained infectious virus (Table 1). However, only 5 of these isolates were able to agglutinate turkey RBCs. Recent studies have shown that some influenza strains bind more effectively to guinea pig RBCs than to turkey RBCs (Barr et al., 2010; Klimov et al., 2012; Nicholls et al., 2008). When guinea pig RBCs were used for agglutination analysis, all 9 of the infectious virus isolates presented agglutination correlated with their measured infectious titers. To determine whether agglutination of each viral isolate was mediated by HA or NA, we repeated agglutination analyses in the presence of 10 μM oseltamivir, a compound that binds in the sialic acid binding site of NA (Collins et al., 2008). Adding in oseltamivir reduced the agglutination of four isolates (Table 1). Surprisingly, only one isolate (Isolate 6) was able to agglutinate turkey RBCs through an NA-independent mechanism. Taken together, these results indicate that 9 of the 10 isolates were able to grow in cell culture, and that 4 of the expanded isolates bind to cells in an NA-dependent mechanism.

Mutations emerge in HA and NA during passage in cell culture

We grouped all the viral isolates into four categories: viruses that agglutinate via an NA-dependent mechanism (n = 4), a virus that agglutinates via an NA-independent mechanism (n = 1), viruses that agglutinate poorly (n = 4), and a virus that was not detected after expansion (n = 1) (Tables 1 and 2). We sequenced the HA1 and NA genes from the primary clinical material and the MDCK-expanded virus of each isolate to identify mutations that may explain the increase in agglutination and the NA-dependent binding. We compared the HA1 and NA sequences to the A/Victoria/361/2011 (A/Victoria/361/11) H3N2 vaccine strain (EpiFlu accession numbers EPI349103 and EPI349104). The primary isolates all contained multiple HA and NA sequence differences compared to the A/Victoria/361/11 vaccine reference strain (Table 2). All four of the viral isolates with NA-dependent agglutination acquired additional mutations at NA residue 151 during expansion, but did not acquire any HA mutations. Previous work in the field has already demonstrated that the two NA mutations we observe here, D151G and D151N, enable NA-dependent viral attachment (Lin et al., 2010; Zhu et al., 2012). Also consistent with previous sequencing studies, we did not detect these NA mutations present at any level in the primary isolate (Lee et al., 2013).

The isolate that agglutinates through an NA-independent manner (Isolate 6) did not acquire any mutations in NA but did acquire a single HA mutation at residue 237 during expansion (Table 2). This isolate also contained one unique mutation, HA F209S, which was present in both the primary sample and the expanded isolate. We did not detect any new HA or NA mutations in the five MDCK-expanded viral isolates that grew to low titers and agglutinated RBCs poorly.

Characterizing the P237L HA mutation

Only the isolate with NA-independent agglutination (Isolate 6) contained mutations at HA residues 209 and 237. To determine if either of these HA mutations (F209S or P237L) was responsible for the increased growth and agglutination of Isolate 6, we completed reverse-genetics studies by inserting F209S and P237L into both the A/Victoria/361/11 H3N2 vaccine strain and Isolate 2. We chose Isolate 2 for reverse-genetics experiments because it agglutinates poorly after expansion and did not acquire any mutations (Tables 1 and 2). We rescued virus containing the mutated HA genes, the NA of either A/Victoria/361/11 or Isolate 2, and the PR8 internal genes by transfecting 293T/MDCK co-cultures. Viruses containing the P237L mutation, but not the F209S mutation, grew to high titers and agglutinated RBCs in the presence of oseltamivir, demonstrating NA-independent binding (Table 3). HA residue 237 is located at the HA trimer interface (Fig. 3A), and thus may contribute to increased growth and agglutination by increasing HA stability. We determined the relative receptor binding avidities of viruses possessing P237 or L237 by measuring agglutination using RBCs pre-treated with receptor-destroying enzyme (RDE) that removes sialic acid receptors (Hensley et al., 2009; Yewdell et al., 1986). The A/Victoria/361/11 virus with the P237L mutation efficiently agglutinated RBCs treated with larger amounts of receptor-destroying enzyme (RDE, a neuraminidase), indicating that viruses with L237 possess higher receptor binding avidities (Fig. 3B).

Previous work has demonstrated that viruses with higher avidity are difficult to inhibit in HAI assays and may result in artificially lower HAI titers even if antigenicity has not changed. To test whether the P237L mutation impacts antigenic analyses, we

conducted an HAI assay using ferret antisera generated against the A/Victoria/361/11 vaccine strain. Addition of the P237L mutation resulted in a twofold decrease in HAI titers compared to the WT virus (Fig. 3C), consistent with the idea that increased receptor binding avidity can alter the interpretation of antigenic analyses.

Although standard Sanger sequencing could not detect the P237L HA mutation in the original clinical material of Isolate 6 (Table 2), low levels of the mutation were detected in the original clinical isolate by sequencing individual clones following TA cloning of PCR products (Fig. 3D). Out of the 21 total clones sequenced, ~5% contained L237. This indicates that the P237L mutation at the HA trimer interface was present in a small subset of the original clinical isolate and was able to grow out during expansion, presumably due to increased HA stability and receptor binding avidity.

DISCUSSION

Taken together, our data indicate that recent clinical H3N2 strains replicate poorly in MDCK cells and rapidly acquire either HA or NA mutations when propagated *in vitro* prior to antigenic testing. These mutations increase viral growth and agglutination titers and can potentially skew HAI assays. The goal of HAI assays is to detect HA antigenic changes, and therefore it is difficult to interpret data when agglutination is mediated by mutated NAs. The addition of oseltamivir directly into HAI assays may circumvent this problem (Lin et al., 2010). We have previously shown that HA mutations that alter receptor binding avidity can skew antigenic maps based on HAI assays (Li et al., 2013a), and recent computational methods have been developed to create antigenic maps that account for variations in viral avidity (Bedford et al., 2014; Li et al., 2013a). Future

studies should focus on alternative cell-derived systems for propagating primary influenza isolates, such as MDCK cells engineered to express different types of sialic acid receptors (Krauss et al., 2012; Oh et al., 2008). Additional studies should also address if cell culture-derived mutations in vaccine strains impact immunogenicity, because recent studies suggest that egg-derived mutations in vaccine strains can have this effect (Skowronski et al., 2014).

In this study, we have displayed the relative ease that recent H3N2 clinical isolates mutate with when propagated in MDCK cell culture. Mutations arising in either HA or NA can alter antigenic properties of these viruses and mask the true identity of the originally collected isolate. Better strategies for expanding these poorly growing influenza isolates and for analyzing their antigenic properties are required in order to ensure accurate viral surveillance and proper selection of vaccine strains.

MATERIALS AND METHODS

Isolate Collection and Expansion

Respiratory secretions were collected from the nasopharnyx of 10 sick children (ages 1 month to 7 years old) at the Children's Hospital of Philadelphia for routine diagnostic purposes during the 2012-2013 influenza season. All samples were tested and confirmed to be infected with H3N2 influenza virus. Leftover, de-identified samples were then sent to us for expansion in MDCK cells, a common cell line used for influenza propagation (Krauss et al., 2012). Isolates were passaged twice on MDCK cells using serum-free Minimum Essential Medium (MEM) containing 0.1% L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin, 0.1% gentamicin, and 0.5% 1

M HEPES buffer. Cell expansions were incubated at 37°C with 5% CO_2 for four days. Supernatant was collected and cell debris was removed via centrifugation at 2500 rpm for 3 min.

Hemagglutination Assays

MDCK-expanded isolates were serially diluted twofold in a 96-well round-bottom plate. Then, 12.5 μL of a 2% (vol/vol) turkey RBC solution or a 2.5% (vol/vol) guinea pig RBC solution were added. Agglutination was read out after incubating for 1 h at room temperature. For hemagglutination assays looking to determine NA-dependent receptor binding, oseltamivir was added to each well at a final concentration of 10 μM.

$TCID_{50}$ Assays

We plated MDCK cells in a 96-well flat-bottom plate at 40000 cells/well in MEM with 9% fetal bovine serum. Next day, cells were washed twice with serum-free MEM before adding 180 μL serum-free MEM containing 0.1% TPCK-treated trypsin, 0.1% gentamicin, and 0.5% 1 M HEPES buffer. MDCK-expanded isolates were then serially diluted tenfold across the plate. Isolates were run in quadruplicate and pipet tips were changed for each row. Plates were incubated for 4 d at 37°C with 5% CO_2. All wells were checked for cytopathic effect and $TCID_{50}$/mL values were calculated.

Sequencing of Isolates

We obtained the HA1 and NA reference sequences of the A/Victoria/361/2011 H3N2 vaccine strain from the EpiFlu database (accession numbers EPI349103 and

EPI349104, respectively). We extracted RNA from the original clinical samples and the MDCK-expanded supernatant using the Qiagen QIAamp Viral RNA Mini Kit. One-Step RT-PCR (Qiagen) using H3N2 HA and NA specific primers was then used to amplify the influenza HA1 and NA genes of interest. After gel purification of PCR products, Sanger sequencing was used to sequence all samples. For Isolate 6, enhanced sequencing of the original clinical sample was performed using TA cloning (TOPO TA Cloning Kit; Life Technologies) of the PCR products. The P237L HA mutation was mapped on the crystallized H3 trimer structure (PDB entry 1HGG).

Reverse-Genetics Derived Viruses

We extracted RNA from the A/Victoria/361/2011 vaccine strain and from Isolate 2, and then cloned the HA and NA of each virus into the pHW2000 reverse genetics plasmid. We inserted the F209S and P237L HA mutations using the QuikChange site-directed mutagenesis kit (Stratagene). We rescued viruses containing the different HA genes, the NA of A/Victoria/361/2011 or Isolate 2, and the internal genes of PR8 by transfecting 293T/MDCK co-cultures as previously described (Hoffmann et al., 2000). All rescued viruses were then passaged two times in MDCK cells and sequence-confirmed for stability. Supernatant collected after the second passage was used for receptor binding avidity and HAI assays.

Receptor Binding Avidity Assays

As previously described (Hensley et al., 2009; Yewdell et al., 1986), guinea pig RBCs were pre-treated with different amounts of RDE for 1 h at 37°C. The RBCs were

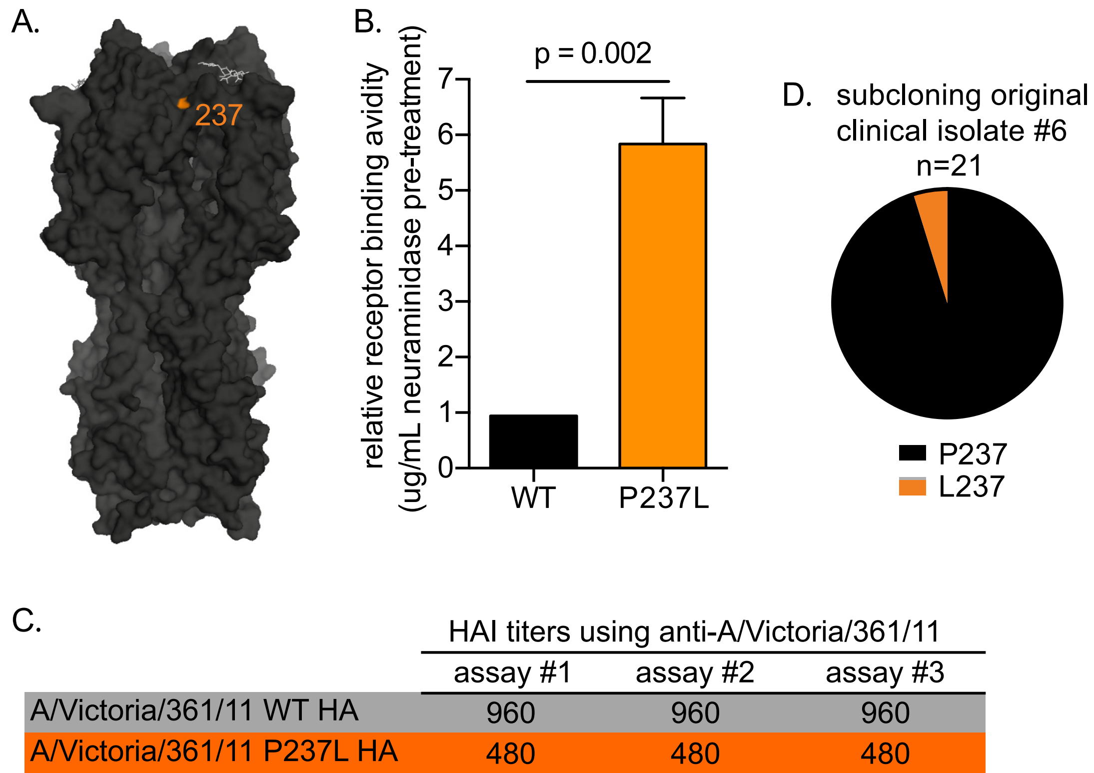
then washed twice with PBS and added as 2% (vol/vol) solutions to four agglutinating doses of each virus (as determined using non-treated guinea pig RBCs). Extent of agglutination was measured after 1 h incubation at room temperature. The viruses with higher receptor binding avidities are able to bind to RBCs that are treated with higher amounts of RDE (Hensley et al., 2009; Yewdell et al., 1986).

Hemagglutination Inhibition Assays with Ferret Antisera

Ferret antiserum to A/Victoria/361/2011 (H3N2), FR-1079, was obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. Antiserum was serially diluted twofold in PBS in a 96-well round-bottom plate. Four agglutinating doses of virus were then added to the diluted antiserum in a total volume of 100 µL. Next, 12.5 µL of a 2.5% (vol/vol) guinea pig RBC solution were added. Agglutination was read out after incubating for 1 h at room temperature. HAI titers are reported as the inverse of the highest dilution that inhibited hemagglutination of guinea pig RBCs.

FIGURES AND TABLES

Figure 3



	HAI titers using anti-A/Victoria/361/11		
	assay #1	assay #2	assay #3
A/Victoria/361/11 WT HA	960	960	960
A/Victoria/361/11 P237L HA	480	480	480

Figure 3. Characterization of the P237L HA mutation.

(A) The location of HA residue 237 is highlighted in orange on the H3 trimer structure and sialic acid is colored in white (PDB entry 1HGG). (B) The relative receptor binding avidities of the A/Victoria/361/11 WT and P237L reverse-genetics derived viruses were determined by agglutination of guinea pig RBCs pretreated with different amounts of the neuraminidase RDE. Data are expressed as the maximum concentration (µg/mL) of neuraminidase that still allowed full agglutination. Means and standard errors of the means of the results for triplicate samples are shown. Data are representative of two

independent experiments. Viruses were compared using one-tailed, unpaired *t* tests. (C) HAI assays comparing the WT and P237L reverse-genetics derived viruses were performed using ferret antisera raised against the A/Victoria/361/11 H3N2 vaccine strain. Experiment was conducted three independent times and all raw HAI titers are displayed. (D) The frequencies of P237 (black) and L237 (orange) were determined after TA cloning of the PCR product amplified from the original clinical material of Isolate 6. A total of 21 clones were sequenced following TA cloning.

Table 1

Isolate no.	$TCID_{50}$/ml	Turkey HAU		Guinea pig HAU		Category
		−Oselt	+Oselt	−Oselt	+Oselt	
1	1.35E + 07	19	<2	48	16	NA dependent
2	4.07E + 06	<2	<2	4	4	Low
3	1.35E + 06	<2	<2	4	3	Low
4	4.07E + 06	<2	<2	6	6	Low
5	<1.00E + 02	<2	<2	<2	<2	Not detected
6	5.56E + 07	36	30	128	128	NA independent
7	6.88E + 07	3	<2	16	8	NA dependent
8	1.89E + 07	22	<2	64	12	NA dependent
9	4.07E + 06	2	<2	8	4	NA dependent
10	1.48E + 06	<2	<2	4	4	Low

Table 1. Infectious and HAU titers of expanded clinical isolates.

Clinical isolates collected from H3N2-infected children at the Children's Hospital of Philadelphia were passaged twice on MDCK cells. Supernatant was collected and tested for infectious titer ($TCID_{50}$/mL) and hemagglutination units (HAU). HAU titers are reported as the maximum dilution of expanded viral isolates that still results in full agglutination of 12.5 μL of 2% (vol/vol) turkey RBCs or 2.5% (vol/vol) guinea pig RBCs. Agglutination was also determined in the presence of 10 μM oseltamivir (Oselt.) to detect NA-dependent binding. Each isolate was placed into one of four categories based on its ability to grow, to agglutinate RBCs, and whether agglutination was NA-dependent or NA-independent.

Table 2

Isolate no.	Category	Differences in NA sequence from that of the H3N2 vaccine strain[b]	NA mutations upon MDCK expansion	Differences in HA sequence from that of the H3N2 vaccine strain[b]	HA mutation upon MDCK expansion
1	NA dependent	V143 M, K258E, T329N	**D151G**	Q49R, N161S, N294K	
7	NA dependent	N141S, K258E, T329N	**D151N**	Q49R, T144A, R158G, N161S, Q189H, N294K	
8	NA dependent	K258E, T329N	**D151G**	Q49R, T144A, R158G, N161S, N294K	
9	NA dependent	K258E, T329N	**D151G**	R158G, N161S, N294K	
6	NA independent	K258E, T329N		Q49R, T144A, R158G, N161S, F209S, N294K	**P237L**
2	Low	K258E, T329N		T144A, R158G, N160S, N161S, N294K	
3	Low	K128R, V143M, K258E, T329N		Q49R, N161S, N294K	
4	Low	P79S, G93D, E221D, K258E, T329N		Q49R, N161S, N294K	
10	Low	K258E, T329N		Q49R, N161S, N294K	

Table 2. Differences in sequences of clinical isolates before and after MDCK expansion.

We sequenced the HA1 and NA genes of each original clinical isolate and twice MDCK-expanded isolate and compared the sequences to the A/Victoria/361/11 H3N2 vaccine strain (EpiFlu accession numbers EPI349103 and EPI349104). Multiple differences in both HA and NA compared to the H3N2 vaccine strain were detected for all original clinical isolates. Upon expansion, mutations arose at NA residue 151 and HA residue 237 (shown in **bold**). Isolates are grouped top to bottom depending on their agglutination "Category" (classifications described in Table 1).

Table 3

Virus	$TCID_{50}$/ml	Turkey HAU		Guinea pig HAU	
		−Oselt	+Oselt	−Oselt	+Oselt
A/Victoria/361/11 WT HA	1.20E + 06	2	2	16	16
A/Victoria/361/11 F209S HA	2.58E + 04	<2	<2	<2	<2
A/Victoria/361/11 P237L HA	1.76E + 07	192	192	256	256
Isolate 2 WT HA	1.20E + 05	<2	<2	3	3
Isolate 2 F209S HA	1.76E + 02	<2	<2	<2	<2
Isolate 2 P237L HA	2.58E + 06	48	48	64	64

Table 3. Infectious and HAU titers of reverse-genetics viruses.

We used reverse genetics to rescue viruses with the WT, F209S, or P237L mutations in either the A/Victoria/361/11 or Isolate 2 HA. Rescued viruses were passaged twice on MDCK cells and sequence confirmed to verify no new mutations had arisen. The infectious titer ($TCID_{50}$/mL) and hemagglutination unit (HAU) titers were determined and reported as in Table 1 above. Viruses possessing the P237L HA mutation are shown to grow to higher infectious titers and exhibit increased agglutination.

CHAPTER 3 – IDENTIFICATION OF THE MUTATIONS RESPONSIBLE FOR THE ANTIGENIC DRIFT OF H3N2 INFLUENZA VIRUSES DURING THE 2014-2015 SEASON

Parts of this chapter were previously published in:

Chambers BS, Parkhouse K, Ross TM, Alby K, and Hensley SE. (2015) Identification of hemagglutinin residues responsible for H3N2 antigenic drift during the 2014-2015 influenza season. *Cell Reports* 12:1-6.

SUMMARY

Influenza vaccines must be updated regularly because influenza viruses continuously acquire mutations in the antibody binding sites of hemagglutinin (HA). The majority of H3N2 strains circulating in the Northern Hemisphere during the 2014-2015 season were antigenically mismatched to the A/Texas/50/2012 H3N2 vaccine strain. H3N2 strains that circulated during that season possessed several new HA mutations that arose after selection of the vaccine strain, and it was unknown which of these mutations contributed to the 2014-2015 vaccine mismatch. Here, we used reverse genetics to demonstrate that mutations in HA antigenic site B were primarily responsible for the vaccine mismatch. Antisera isolated from vaccinated humans and infected animals had

reduced hemagglutination inhibition and *in vitro* neutralization titers against reverse-genetics-derived viruses possessing mutations in 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 supported the World Health Organization's decision to update the H3N2 component of the vaccine for the 2015-2016 influenza season.

INTRODUCTION

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

H3 HAs have at least five distinct antigenic sites, named HA antigenic sites A-E (Wiley et al., 1981). Seasonal influenza vaccine strains are routinely chosen based on antigenic analyses that utilize antisera prepared in previously naïve ferrets (Stöhr et al., 2012). Koel and colleagues recently demonstrated that most Ab responses from

previously naïve ferrets infected a single time with an H3N2 virus 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 HA antigenic site A of some H3 strains (Abe et al., 2004) and on HA antigenic site B of other H3 strains (Popova et al., 2012).

It is important to determine which HA residues were 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 were present in H3N2 strains that circulated during the 2014-2015 influenza season. We found 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 (WHO) recommended that the H3 component of seasonal influenza vaccines should be updated to include A/Switzerland/9715293/2013-like strains (Anonymous, 2015). Our data supported this recommendation, although we note that the majority of H3N2 strains circulating at the time had a distinct HA antigenic site B compared to the A/Switzerland/9715293/2013 strain.

RESULTS

2014-2015 H3N2 viruses possessed several HA mutations

The H3N2 component of the 2014-2015 influenza vaccine was A/Texas/50/2012, which belongs to HA genetic clade 3C.1 (Broberg et al., 2015). During the 2014-2015 season, H3N2 strains belonging to phylogenetic HA clades 3C.2a, 3C.3, and 3C.3a predominated (Broberg et al., 2015). Compared to the A/Texas/50/2012 vaccine strain, clade 3C.2a viruses possessed HA differences L3I, N144S, N145S, F159Y, K160T, N225D, and Q311H; clade 3C.3 viruses possessed HA differences T128A, R142G, and N145S; and clade 3C.3a viruses possessed HA differences T128A, A138S, R142G, N145S, F159S, and N225D (Table 4). HA clade 3C.2a and 3C.3a viruses were antigenically distinct compared to the A/Texas/50/2012 strain and the WHO recommended that the H3N2 component should be updated with an A/Switzerland/9715293/2013-like (HA clade 3C.3a) virus for the Northern Hemisphere 2015-2016 vaccine (Anonymous, 2015). Analyses of HA sequences deposited in the Global Initiative on Sharing All Influenza Data (GISAID) database revealed that the majority of H3N2 viruses circulating in the United States in the fall and winter of 2014 belonged to HA clade 3C.2a (Fig. 4A). We performed a similar analysis for Philadelphia-area H3N2 viruses using clinical isolates collected from the Hospital of the University of Pennsylvania. Primary isolates were directly sequenced and revealed a similar distribution of circulating genetic clades during the 2014-2015 influenza season as compared to the GISAID database sequences (Fig. 4B).

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 the HA mutations found in HA clade 3C.2a, 3C.3, and 3C.3a viruses (Table 5). We then completed antigenic analyses using antisera isolated from ferrets, sheep, and humans exposed to the A/Texas/50/2012 H3N2 vaccine strain (Tables 6 and 7). Our mutant viral panel included every mutation from Table 4 that is located in the HA globular head (Fig. 4C), 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 (RBCs) and quickly mutated at HA residue 160 when propagated for antigenic analyses. However, we were still able to assess the antigenic relevance of mutations in this epitope of HA because 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 antisera collected from ferrets and sheep recovering from primary A/Texas/50/2012 infections (Table 6). 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 reduced fourfold in HAI assays against viruses engineered to possess the F159S HA mutation (Table 6). HA residue 159 is located in a highly exposed region of HA antigenic site B in H3 HAs (Fig. 4C). 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 twofold decreases in HAI titers. We have previously shown that viruses that bind to RBCs with a high avidity can escape Abs in HAI assays independent of antigenic

change (Li et al., 2013a). We found that A138S and N145S HA mutant viruses bound to RBCs with a higher avidity (Table 5), and the apparent decrease in HAI titer using these viruses likely results from this increase in receptor binding avidity. We found that viruses possessing the F159S HA mutation bound to RBCs with a decreased avidity (Table 5), 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 direct Ab ELISA binding assays with plates coated with virus-like particles (VLPs) possessing A/Texas/50/2012-WT HA or A/Texas/50/2012-F159S HA (Fig. 5). As a control, we coated plates with VLPs possessing the antigenically distinct A/Port Chalmers/1/1973 HA (an H3N2 virus from 1973) as well. Antisera 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 (Fig. 5A). 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 (Fig. 5B). Collectively, these studies indicate that ferrets and sheep mount Ab responses against an HA epitope involving HA 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 the HA epitope involving HA residue 159

We also completed HAI assays using our mutant viral panel and antisera isolated

from sheep infected with A/Switzerland/9715293/2013 H3N2 virus. Antisera isolated from A/Switzerland/9715293/2013-infected animals reacted to all mutant A/Texas/50/2012 viruses, including viruses that possessed the HA antigenic site B F159S mutation (Table 6). Therefore, the F159S HA mutation results in an asymmetrical antigenic change. These data are important because A/Switzerland/9715293/2013 (an HA clade 3C.3a virus) was chosen as the H3 component of 2015-2016 seasonal vaccines (Anonymous, 2015), even though HA clade 3C.2a viruses predominated toward the end of the 2014-2015 influenza season (Fig. 4A) and have continued to dominate circulation since. And although HA antigenic site B differs between HA clade 3C.3a and 3C.2a viruses (Table 4), these initial results indicated that vaccinating with an A/Switzerland/9715293/2013-like virus should elicit an Ab response effective against the HA clade 3C.2a viruses dominating circulation.

Antisera isolated from most humans vaccinated with A/Texas/50/2012 possess Abs against HA antigenic site B

Next, we wanted to determine whether human Abs elicited against A/Texas/50/2012 have the same specificity for HA antigenic site B as we observed in ferret and sheep antisera. We completed HAI assays using the A/Texas/50/2012-WT and A/Texas/50/2012-F159S viruses, along with human sera collected pre- and post-vaccination with the 2014-2015 seasonal influenza vaccine, which contained the A/Texas/50/2012 H3N2 vaccine strain. Even before vaccination, over half of the individuals presented detectable titers against the A/Texas/50/2012-WT virus (Table 7). This is likely because the A/Texas/50/2012 strain had been in seasonal influenza vaccines

since the 2013-2014 season and thus many people would have been exposed to this strain previously. Following vaccination, most individuals (87.5%) either mounted or maintained Abs that had reduced reactivity against the A/Texas/50/2012-F159S mutant virus (Table 7). Even in a cohort that covers a fairly large age range (21-50 years old), the majority of individuals mounted anti-A/Texas/50/2012 Ab responses focused on HA antigenic site B. It is important to point out however, that some individuals (12.5%) in this cohort did not possess detectable levels of Abs against HA antigenic site B although they mounted high responses against the A/Texas/50/2012-WT virus (Table 7). It is possible that these individuals have an Ab response targeted against another HA antigenic epitope due to unique pre-exposure histories that shaped their Ab response, similar to what we have seen previously in certain age groups that possess altered immunodominance of H1N1 Ab responses (Li et al., 2013b; Linderman et al., 2014).

Anti-A/Texas/50/2012 HA antigenic site B Abs are neutralizing

Although we established that the majority of ferret, sheep, and human Abs against A/Texas/50/2012 targeted HA antigenic site B, it wasn't known whether these Abs were neutralizing. To determine the functional consequence of the F159S HA mutation, we completed *in vitro* neutralization assays with A/Texas/50/2012-WT and A/Texas/50/2012-F159S viruses 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-WT and A/Texas/50/2012-F159S (Table 7). We also completed *in vitro* neutralization assays with antisera collected from ferrets 19 days after infection with A/Texas/50/2012. Overall, the *in vitro* neutralization data mirrored our HAI results, with

all seven human sera samples and the ferret sera sample presenting dramatically reduced neutralization titers against the A/Texas/50/2012-F159S virus compared to the A/Texas/50/2012-WT virus (Table 8). These results indicate that the anti-A/Texas/50/2012 Abs targeted against HA antigenic site B in humans and ferrets are neutralizing, and that a single mutation at HA residue F159 can abrogate neutralization by these Abs.

DISCUSSION

There was a clear H3N2 vaccine mismatch during the 2014-2015 influenza season (Broberg et al., 2015; D'Mello et al., 2015a; Flannery et al., 2015; Pebody et al., 2015), and it is important to identify the specific HA mutations that led to this mismatch. This information could be crucial for properly selecting viral strains to be used in future vaccine formulations. In this report, we demonstrated 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 differences in HA antigenic site B compared to the vaccine strain (Fig. 4).

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 old humans mount anti-

A/Texas/50/2012 Abs against HA antigenic site B following vaccination. It is important to note that some vaccinated individuals in our study (4 out of 32) mounted anti-A/Texas/50/2012 Ab responses that were not directed against HA antigenic site B (antisera from these four individuals had <twofold change in HAI titer using the A/Texas/50/2012-WT and A/Texas/50/2012-F159S viruses). Current studies are under way to investigate whether these four individuals have evidence of unique H3N2 exposures.

The WHO 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 (Fig. 4). Clade 3C.2a viruses possess an additional predicted glycosylation site in HA antigenic site B due to S159Y and K160T HA 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 would 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 HA antigenic site B of 2014-2015 H3N2 strains 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 WHO's decision to update the H3N2 component of the 2015-2016 influenza vaccine.

MATERIALS AND METHODS

HA Sequences

We obtained HA sequence data from the GISAID website http://platform.gisaid.org/epi3/. We grouped all human isolates collected by United States surveillance labs between June 1, 2014 and January 31, 2015 into clades based on HA genetic sequence. All isolates from the Hospital of the University of Pennsylvania were sequenced and compared to the HA sequence of A/Texas/50/2012, EpiFlu accession number EPI408131.

Clinical Isolates

Respiratory secretions were collected from H3N2-infected patients at the Hospital of the University of Pennsylvania between December 2014 and January 2015. RNA was extracted directly from clinical isolates using the Qiagen QIAamp Viral RNA Mini Kit and the HA1 gene was amplified via RT-PCR using H3-specific primers. Sequences were aligned to A/Texas/50/2012 for comparison of HA1 sequence differences.

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. Because 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 QuikChange site-directed mutagenesis kits (Stratagene) to convert the A/Victoria/361/2011 HA sequence to the A/Texas/50/2012 HA sequence (by adding T128N, G186V, S198P, S219F, N278K HA mutations). We then introduced the additional HA mutations from Table 5 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 PR8 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 antisera 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. Antisera were also collected from humans prior to vaccination (d0) 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 (VGTI). All sera were treated with receptor-destroying enzyme (RDE) for 3 h prior to antigenic testing.

HAI Assays

HAI titrations were performed in 96-well round-bottom plates. Sera were serially diluted twofold 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 RBC solution were added. Agglutination was read out after incubating for 1 h at room temperature. HAI titers were recorded as the inverse of the highest dilution that inhibited hemagglutination of turkey RBCs. Similar results were obtained using guinea pig RBCs.

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, PR8 NA, HAT (a 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 immunoglobulin 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 twofold and then added to 100 $TCID_{50}$ 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 h at 37°C. Next, cells were washed, and then serum-free media with TPCK-treated trypsin, HEPES, and gentamicin 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 (Hensley et al., 2009; Li et al., 2013a), turkey RBCs were pre-treated with different amounts of RDE (a neuraminidase) for 1 h at 37°C. The RBCs were washed with PBS and added (as 2% v/v solutions) to four agglutinating doses of each virus (as determined using non-treated RBCs). After incubating 1 h at room temperature, agglutination was measured. Viruses with higher receptor binding avidities are able to bind to RBCs that are treated with higher amounts of RDE (Hensley et al., 2009; Li et al., 2013a; Yewdell et al., 1986).

FIGURES AND TABLES

Figure 4

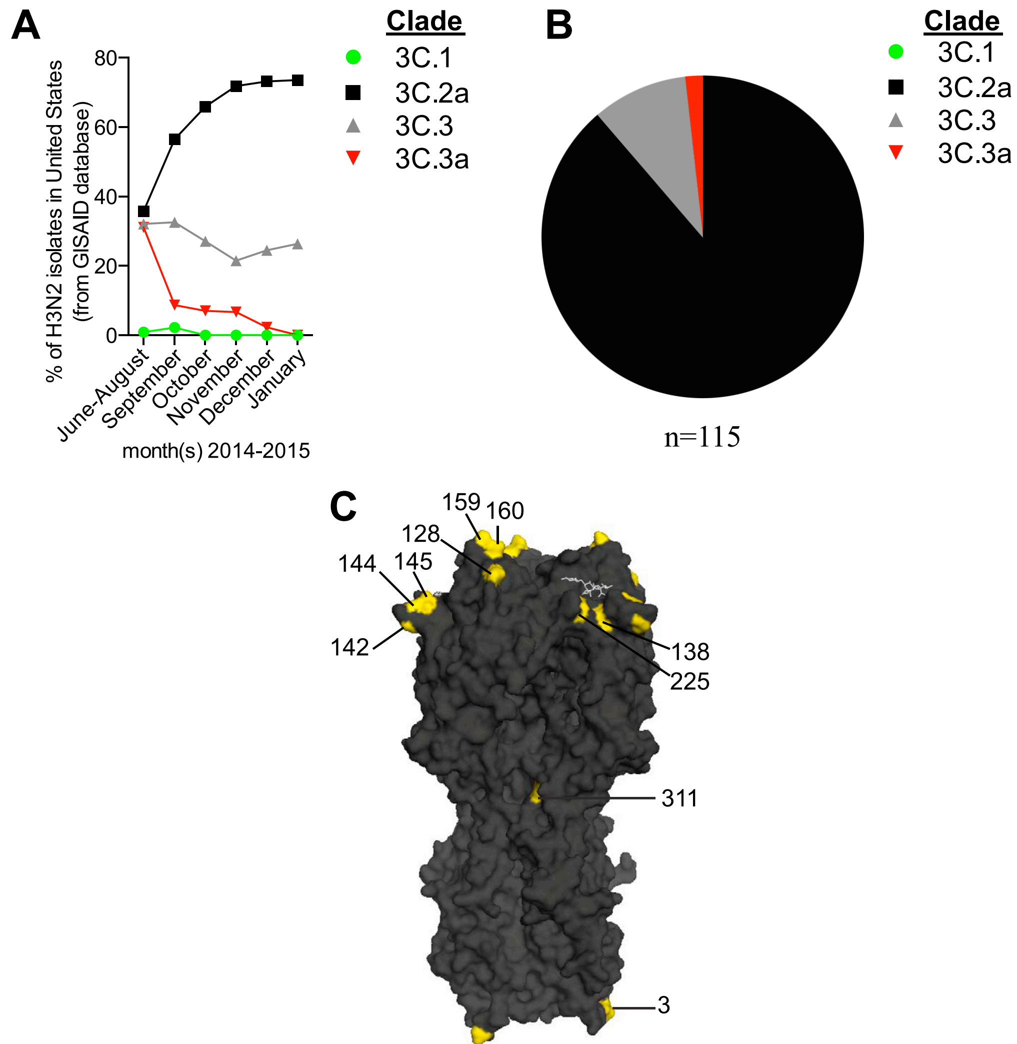


Figure 4. Genetically distinct H3N2 viruses circulated during the 2014–2015 influenza season in the United States.

(A) HA sequences deposited on the GISAID database were analyzed. All sequences deposited by United States laboratories between June 2014 and January 2015 were included in the analysis. Shown are the percent of viruses that belong to each HA clade

(as defined in the text). (B) Clinical isolates collected from patients at the Hospital of the University of Pennsylvania (December 2014-January 2015) were sequenced and compared to the HA sequence of A/Texas/50/2012 (EpiFlu accession number EPI408131). Shown is the fraction of clinical isolates that belong to each HA clade (as defined in the text). (C) The H3 structure (PDB entry 1HGG) is shown with residues from Table 4 highlighted in yellow and labeled.

Figure 5

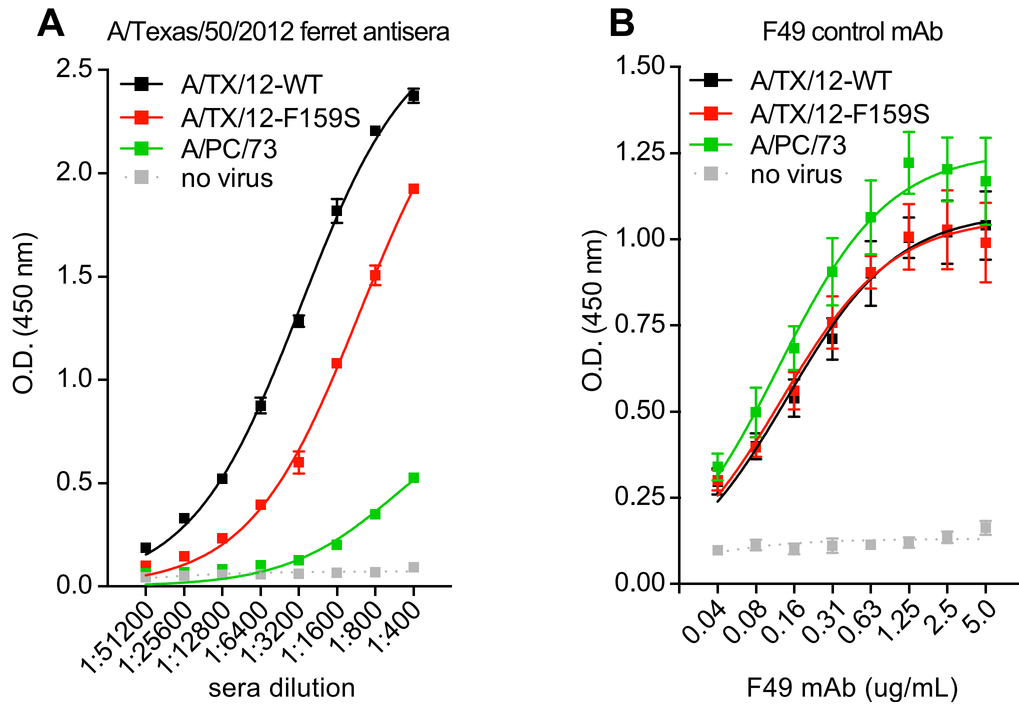


Figure 5. ELISA experiments confirm that the F159S HA mutation abrogates binding of Abs in anti-A/Texas/50/2012 ferret antisera.

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 antisera (A) or the F49 mAb (B) 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.

Table 4

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

Table 4. HA sequence differences between newly emerged clades and the A/Texas/50/2012 vaccine strain.

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

Table 5

viruses	relative receptor binding avidity
A/Texas/50/2012-WT	++
A/Texas/50/2012-N128A	++
A/Texas/50/2012-A138S	+++
A/Texas/50/2012-R142G	++
A/Texas/50/2012-N144S+N145S	++
A/Texas/50/2012-N145S	+++
A/Texas/50/2012-F159S	+
A/Texas/50/2012-N225D	+++
A/Switzerland/9715293/2013	+++

Table 5. Our panel of A/Texas/50/2012 mutant viruses and their relative binding avidities.

Shown is the panel of reverse-genetics derived mutant viruses that we created for our antigenic analyses. Relative receptor binding avidity is also shown (+++ indicates virus was able to agglutinate RBCs treated with > 1.0 ug/mL RDE; ++ indicates virus was able to agglutinate RBCs treated with 0.5-1.0 ug/mL RDE; + indicates virus was able to agglutinate RBCs treated with <0.5 ug/mL RDE). Data are representative of two independent experiments.

Table 6

Viruses	Sera		
	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

Table 6. Analyses of ferret and sheep antisera raised against the A/Texas/50/2012 and A/Switzerland/9715293/2013 vaccine strains.

HAI assays were completed using the A/Texas/50/2012-WT virus and the panel of A/Texas/50/2012 mutant viruses, as well as the A/Switzerland/9715293/2013 WT (HA clade 3C.3a) virus as a control. Antisera were collected from ferrets 19 days post-infection and from sheep 28 days post-infection. Data are representative of three independent assays.

Table 7

Sample ID	Age (years)	Pre-vaccination		Post-vaccination	
		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

Table 7. Analyses of sera isolated from humans pre- and post-vaccination with the 2014-2015 seasonal influenza vaccine.

HAI assays were completed using the A/Texas/50/2012-WT and A/Texas/50/2012-F159S viruses. Sera were collected from 32 humans pre-vaccination (d0) and post-vaccination (d21) with the 2014-2015 seasonal influenza vaccine that contained the A/Texas/50/2012 vaccine strain. Data are representative of three independent assays.

Table 8

	A/Texas/50/2012-WT	A/Texas/50/2012-F159S
human ID 06	640	80
human ID 14	1280	80
human ID 20	640	<40
human ID 21	320	40
human ID 22	640	160
human ID 23	320	80
human ID 31	320	80
ferret anti-A/Texas/50/2012	1280	40

Table 8. *In vitro* neutralization titers using antisera isolated from vaccinated humans and infected ferrets.

In vitro neutralization assays were completed in MDCK cells using the A/Texas/50/2012-WT and A/Texas/50/2012-F159S viruses. Neutralization titers were determined for antisera isolated 21 days following vaccination of humans with the 2014-2015 influenza vaccine or antisera isolated 19 days following infection of ferrets with the A/Texas/50/2012 strain. Human sera samples used here displayed the largest reduction in HAI titers due to the F159S HA mutation (Table 7). Neutralization titers are expressed as the inverse of the maximum dilution of sera that results in full neutralization of viral growth. Data are representative of two independent assays.

CHAPTER 4: POTENTIAL ANTIGENIC EXPLANATION FOR ATYPICAL H1N1 INFECTIONS AMONG MIDDLE-AGED ADULTS DURING THE 2013-2014 INFLUENZA SEASON

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SUMMARY

Influenza viruses typically cause the most severe disease in children and elderly individuals. However, H1N1 viruses disproportionately affected middle-aged adults during the 2013-2014 influenza season. Although H1N1 viruses had recently acquired several mutations in the hemagglutinin (HA) glycoprotein, classic serological tests used by surveillance laboratories indicated that these mutations did not change antigenic properties of the virus. Here, we show that one of these mutations was located in a region

of HA targeted by antibodies elicited in many middle-aged adults. We found that over 42% of individuals born between 1965 and 1979 possessed antibodies that recognize this region of HA. Our findings offer a possible antigenic explanation of why middle-aged adults were highly susceptible to H1N1 viruses during the 2013-2014 influenza season. Our data further suggest that a drifted H1N1 strain should be included in future influenza vaccines to potentially reduce morbidity and mortality in this age group.

INTRODUCTION

Seasonal H1N1 (sH1N1) viruses circulated in the human population for much of the last century and, as of 2009, most humans had been exposed to sH1N1 strains. In 2009, an antigenically distinct H1N1 strain began infecting humans and caused a pandemic (Dawood et al., 2009; Garten et al., 2009; Smith et al., 2009). Elderly individuals were less susceptible to 2009 pandemic H1N1 (pH1N1) viruses because of cross-reactive antibodies (Abs) elicited by infections with older sH1N1 strains (Garten et al., 2009; Jacobs et al., 2012; Manicassamy et al., 2010; Skountzou et al., 2010; Xu et al., 2010). pH1N1 viruses have continued to circulate on a seasonal basis since 2009. Influenza viruses typically cause a higher disease burden in children and elderly individuals (Mertz et al., 2013), but pH1N1 viruses caused unusually high levels of disease in middle-aged adults during the 2013-2014 influenza season (Arriola et al., 2014; Ayscue et al., 2014; Davila et al., 2014; Epperson et al., 2014). For example, a significantly higher proportion of individuals aged 30-59 years old were hospitalized in Mexico with laboratory-confirmed pH1N1 cases in 2013-2014 relative to 2011-2012 (Davila et al., 2014).

Most neutralizing influenza Abs are directed against the hemagglutinin (HA) glycoprotein. International surveillance laboratories rely primarily on ferret antisera for detecting HA antigenic changes (Stöhr et al., 2012). For these assays, sera are isolated from ferrets recovering from primary influenza infections. Seasonal vaccine strains are typically updated when human influenza viruses acquire HA mutations that prevent the binding of sera collected from previously naïve ferrets infected with influenza for the first time. Our laboratory and others have demonstrated that sera isolated from ferrets recovering from primary pH1N1 infections are dominated by Abs that recognize an epitope involving HA residues 156, 157, and 158 of the Sa HA antigenic site (Chen et al., 2010b; Li et al., 2013b). The pH1N1 component of the seasonal influenza vaccine has not been updated since 2009 because very few pH1N1 isolates possess mutations in HA residues 156, 157, and 158. The majority of isolates from the 2013-2014 season have been labeled as antigenically similar to the A/California/07/2009 vaccine strain (Arriola et al., 2014).

It is potentially problematic that major antigenic changes of influenza viruses are mainly determined using antisera isolated from ferrets recovering from primary influenza infections. Unlike experimental ferrets, humans are typically reinfected with antigenically distinct influenza strains throughout their life (Miller et al., 2013). In the 1950s, it was noted that the human immune system preferentially mounts Ab responses that cross-react to previously circulating influenza strains, at the expense of new Ab responses that exclusively target newer viral strains (Davenport et al., 1953). This process, which Thomas Francis, Jr. termed “original antigenic sin,” has been experimentally recapitulated in ferrets (Li et al., 2013b; Webster, 1966), mice (Kim et al., 2009;

Virelizier et al., 1974a; Virelizier et al., 1974b), and rabbits (Fazekas de St and Webster, 1966b). Our group and others recently demonstrated that the specificity of pH1N1 Ab responses can be shaped by prior sH1N1 exposures (Carter et al., 2013; Li et al., 2012; Li et al., 2013b; Pica et al., 2012; Wrammert et al., 2011). We found that ferrets sequentially infected with sH1N1 and pH1N1 viruses mount Ab responses dominated against epitopes that are conserved between the viral strains (Li et al., 2013b). These studies indicate that sera collected from previously naïve ferrets infected a single time with influenza may not be fully representative of human influenza immunity.

It has been proposed that increased morbidity and mortality of middle-aged adults during the 2013-2014 influenza season was primarily a result of low vaccination rates within these populations (Catania et al., 2014). An alternative explanation is that pH1N1 strains that circulated during that season had acquired a true antigenic mutation that was mislabeled as "antigenically neutral" by assays that relied on sera collected from previously naïve ferrets infected a single time with the 2009 pH1N1 virus. Here, we completed a series of experiments to determine if pH1N1 strains that circulated during the 2013-2014 influenza season possessed a mutation that prevented binding of Abs in middle-aged humans who had been previously exposed to different sH1N1 strains.

RESULTS

Recent pH1N1 strains possess a mutation that prevents binding of human Abs

Antisera isolated from ferrets recovering from primary pH1N1 infections are highly specific for an epitope involving HA residues 156, 157, and 158 of the Sa antigenic site of H1 viruses (Chen et al., 2010b; Li et al., 2013b). Very few pH1N1

isolates possessed mutations in these Sa residues as of 2014 (Fig. 6); however, pH1N1 viruses recently acquired a K166Q HA mutation, which is located at the interface of the Sa/Ca antigenic sites (Caton et al., 1982) (Fig. 7A). The K166Q HA mutation first arose during the 2012–2013 season and was present in over 99% of pH1N1 isolates during the 2013-2014 season (Fig. 7B-C). Based on experiments using antisera isolated from ferrets infected for the first time, surveillance laboratories reported that pH1N1 viruses with the K166Q HA mutation were antigenically indistinguishable from the A/California/07/2009 pH1N1 vaccine strain (Arriola et al., 2014).

To address if human Abs are capable of recognizing pH1N1 viruses with the K166Q HA mutation, we performed hemagglutination inhibition (HAI) assays using sera from healthy humans collected during the 2013–2014 influenza season in the United States. Remarkably, 27% of sera from individuals born between 1940 and 1984 possessed Abs specific for an epitope involving HA residue K166 (Fig. 8A and Table 9). Over 42% of individuals born from 1965 to 1979 had K166 HA-specific Abs in their sera ($n = 54$ individuals). Sera isolated from individuals born between 1985 and 1997 ($n = 49$ individuals) did not have detectable levels of K166 HA-specific Abs. Differences in K166 HA-specificity were statistically significant between sera isolated from individuals born between 1965 and 1979 and individuals born after 1985 (Fisher's exact test; $P < 0.0001$). Similar results were obtained when we analyzed sera from healthy humans collected during the 2013–2014 influenza season in Mexico (Fig. 9 and Table 10).

It is remarkable that HAI assays, which are relatively insensitive, were able to reproducibly detect K166 HA-specific Abs in so many individuals in our experiments. Fig. 8A and Fig. 9 show percentages of donors that had at least a twofold reduction in

HAI titer using the K166Q HA mutant virus in three independent assays. It is worth pointing out that many sera samples had over fourfold reduced HAI titers using a pH1N1 virus engineered to possess the single K166Q HA mutation compared with the pH1N1 vaccine strain (Table 9). Age-related differences in K166 HA-specificity among United States donors remained statistically significant using a fourfold reduction in HAI titer as a cut-off (Fisher's exact test; $P < 0.05$ comparing donors born between 1965 and 1979 and donors born after 1985). Sera that had K166 HA-specificity based on HAI assays failed to efficiently neutralize K166Q-possessing viruses in *in vitro* neutralization assays as well (Table 11). K166 HA-specific sera were also unable to recognize a primary viral isolate collected in 2013 (A/CHOP/1/2013) that possesses the K166Q HA mutation (Table 11).

A glycosylation site present in sH1N1 viruses circulating after 1985 shields the K166 HA-epitope

Original antigenic sin Abs are originally primed by influenza strains that circulated in the past (Davenport et al., 1953; Fazekas de St and Webster, 1966a, b; Li et al., 2013b; Webster, 1966). We propose that K166 HA-specific Ab responses were likely primed by sH1N1 viruses circulating in humans before 1985 and then boosted by the 2009 pH1N1 virus. Sera isolated from individuals born between 1965 and 1979 had the highest K166 HA-specificity (both in percent and in titer) (Fig. 8A and Table 9). sH1N1 viruses that circulated in the late 1970s and early 1980s share extensive homology with pH1N1 viruses in the vicinity of K166 (Fig. 8B). sH1N1 viruses were absent from the human population from 1957 to 1976 and began infecting humans again in 1977.

Therefore, humans born between 1957 and 1976 likely had their first H1N1 encounter with a sH1N1 virus that shared homology with pH1N1 viruses in the vicinity of K166.

In 1986, sH1N1 viruses acquired a new glycosylation site at HA residue 129 that is predicted to shield the epitope involving K166 (Figs. 10 and 11). The absence of K166 HA-specific Ab responses in individuals born after 1985 is likely because sH1N1 viruses glycosylated at HA residue 129 fail to prime K166 HA-specific responses. The lower number of K166 HA-specific responders born in the 1950s might also be attributed to unique glycosylation sites in sH1N1 viruses that circulated during this time period (Fig. 10), although precise glycosylation statuses of viruses circulating before 1977 are uncertain because of limited numbers of sequenced viruses. Although we did not examine sera from very elderly individuals, it is possible that they also have immunodominant K166 HA-specific responses, because a recent study reported that a mAb isolated from a survivor of the 1918 H1N1 pandemic binds to pH1N1 in an epitope involving K166 (Xu et al., 2010). There is considerable homology between the 1918 H1N1 virus and the 2009 pH1N1 virus in the vicinity of K166 (Xu et al., 2010).

To experimentally address if glycosylation sites present in previous sH1N1 strains shield the epitope involving K166, we used reverse-genetics to produce pH1N1 viruses that had glycosylation sites that were either present in sH1N1 strains from 1977-1985 (sites 131+163) or 1986-2008 (sites 129+163). Western blot analysis revealed that HA residues 129 and 131, but not HA residue 163, were glycosylated in our reverse-genetics derived viruses (Fig. 11B). Consistent with the hypothesis that the K166 HA-epitope is shielded by glycosylation sites present in 1986-2008 sH1N1 viruses, K166 HA-specific human sera had reduced titers to pH1N1 viruses with the 129-glycosylation site, but

normal titers to pH1N1 viruses with the 131-glycosylation site (Fig. 11C). As a control, we also completed HAI assays with sera from donors that were born in the 1970s who did not have detectable levels of K166 HA-specific sera Abs. As expected, these sera did not have reduced titers to pH1N1 viruses with the 129-glycosylation site, but interestingly, these sera did have reduced titers to pH1N1 viruses with the 131-glycosylation site (Fig. 11C). We previously demonstrated that Ab responses focused on an epitope near the 131-glycosylation site can be elicited by sequential infections with a sH1N1 virus from the early 1990s and the pH1N1 virus (Li et al., 2013b). We speculate that donors in the "non-K166 HA-specific" group were previously infected with antigenically distinct sH1N1 strains compared with donors in the K166 HA-specific group (ie: A/Singapore/06/1986-like strain instead of A/Chile/01/1983-like strain). Taken together, these data suggest that glycosylation sites on previously circulating sH1N1 viruses shield epitopes and influence the development of subsequent Ab responses against pH1N1 virus.

Vaccination with current pH1N1 vaccine strain elicits K166 HA-specific Abs

The pH1N1 vaccine strain has not been updated since 2009. We determined whether this vaccine strain, which possesses an HA with K166, elicits K166 HA-specific Abs in humans. First, we analyzed sera from individuals vaccinated in 2009. All of the individuals in this cohort were born before 1984 and most did not have pH1N1 Ab titers before vaccination (Table 12). Sera from 5 of 17 individuals possessed detectable levels of K166 HA-specific Abs following vaccination (Fig. 12 and Table 12). Sera from all five of these individuals had <1:40 HAI titers against the K166Q HA mutant pH1N1 virus (Table 12). One K166 HA-specific individual (subject #1) possessed K166 HA-

specific Abs before vaccination (Fig. 12A and Table 12). It is possible that this individual was naturally infected with pH1N1 before vaccination. All of the K166 HA-specific donors had detectable pre-vaccination Ab titers against sH1N1 viruses from 1977 and 1983; however, we also found titers against these strains in some donors that did not have detectable levels of K166 HA-specific serum Abs (Table 12). We also measured binding of 42 HA head-specific monoclonal Abs (mAbs) isolated from 12 adult donors (born 1949-1985) that were vaccinated against the pH1N1 strain in 2009. Strikingly, 23% of these mAbs had reduced binding to pH1N1 engineered to have the K166Q mutation (Fig. 12B). This finding is consistent with a previous report that identified several K166 HA-specific mAbs derived from a donor that was born before 1977 (Krause et al., 2011).

We passively transferred a K166 HA-specific mAb (SFV009-3F05) or a control mAb that binds equally to WT and K166Q-HA pH1N1 viruses (SFV015-1F02) to BALB/c mice 12 h before infecting them with a lethal dose of WT or K166Q-HA pH1N1 viruses. Control animals that did not receive a mAb before infection rapidly lost weight and died or needed to be euthanized (Fig. 12C). Mice receiving the control SFV015-1F02 mAb before infection with WT or K166Q-HA pH1N1 viruses all survived with minimal weight loss (Fig. 12C). Mice receiving the K166 HA-specific SFV009-3F05 mAb survived following infection with WT pH1N1 but rapidly lost weight and died or needed to be euthanized following infection with K166Q-HA pH1N1 (Fig. 12C). These data suggest that K166 HA-specific Abs can be less efficient at preventing disease in a mouse model following infection with a pH1N1 virus possessing K166Q HA.

Can K166 HA-specific immunity be recapitulated in ferrets for surveillance purposes?

Current surveillance efforts rely heavily on antisera isolated from ferrets recovering from primary influenza virus infections. Ferret antisera could potentially be more reflective of human immunity if isolated from animals sequentially infected with antigenically distinct viral strains. We attempted to elicit K166 HA-specific Abs in ferrets by sequentially infecting animals with older sH1N1 strains and then the A/California/07/2009 pH1N1 strain.

We initially infected animals with a sH1N1 virus that circulated in 1977 (A/USSR/90/1977), a sH1N1 virus that circulated in 1983 (A/Chile/01/1983), or a sH1N1 virus that circulated in 1986 (A/Singapore/06/1986). After 84 d, we re-infected animals with the A/California/07/2009 pH1N1 strain. As controls, we infected some animals twice with A/California/07/2009 and other animals only once with A/California/07/2009. Three of eight ferrets sequentially infected with A/Chile/01/1983 and A/California/07/2009 mounted K166 HA-specific Abs detectable in HAI assays (Fig. 13 and Table 13). The 22 ferrets in the other experimental groups did not mount detectable levels of K166 HA-specific Abs. The difference in K166 HA-specificity is statistically significant comparing the A/Chile/01/1983-A/California/07/2009 group with the rest of the groups (3 of 8 vs. 0 of 22; Fisher's exact test $P < 0.05$). K166 HA-specific Abs were likely not elicited in the A/Singapore/06/1986-A/California/07/2009 group because the K166 HA-epitope is predicted to be shielded by a glycosylation site at HA residue 129 of A/Singapore/06/1986 (Fig. 11). It is interesting that K166 HA-specific Abs were not elicited by A/USSR/90/1977-A/California/07/2009 sequential infections. This result is likely because of variation at HA residue 125, which is close to HA residue 166 (Fig. 14).

A/Chile/01/1983 and A/California/07/2009 both possess S125, whereas A/USSR/01/1977 possesses R125 (Fig. 14).

New pH1N1 vaccine strain candidate is antigenically distinct compared to A/California/07/2009

Although the pH1N1 vaccine strain component has not been updated since 2009, a new strain was added to the list of "A/California/07/2009-like" candidate strains for the upcoming 2016-2017 influenza season (Anonymous, 2016). Despite being labeled as antigenically identical to the A/California/07/2009 vaccine strain, the new strain, A/South Africa/3626/2013, possesses the HA K166Q mutation compared to the current vaccine strain. This is the first virus added to list of H1N1 candidate vaccine strains that possesses a glutamine at HA residue 166. Based on our previous data indicating that a large percentage of middle-aged adult human sera possess Abs that are sensitive to the HA K166Q mutation, we hypothesized that A/South Africa/3626/2013 is actually an antigenically distinct virus compared to A/California/07/2009. We generated both the A/California/07/2009 X-179A (Cal X-179A) and A/South Africa/3626/2013 X-243 (S.A. X-243) vaccine strains using reverse genetics and PR8 internals. After rescue, the viruses were expanded in eggs similar to how the vaccine strains are produced.

We then performed HAI assays using antisera collected from middle-aged adults (born 1977-1985), both before (d0) and after (d21) vaccination with A/California/07/2009, during the 2013-2014 influenza season (Fig. 15). Even before vaccination, the majority of the 20 individuals from the cohort had anti-pH1N1 Ab titers. Of the 14 samples with titers against Cal X-179A, 36% had HAI titers reduced at least

twofold against S.A. X-243 (Fig. 15A). Following vaccination, the percentage of individuals with at least a twofold reduction in HAI titer against S.A. X-243 was 37% (Fig. 15B). These results are consistent with data from Fig. 8A, where the sera from 42% of the middle-aged adults (born 1965-1979) in our cohort had reduced HAI titers against a virus containing the K166Q HA mutation. Relative receptor binding avidities of the two viruses were calculated to have no significant difference (Fig. 15C), indicating that observed changes in HAI titers were likely due to antigenic differences.

Overall, the HAI data confirms our hypothesis that A/South Africa/3626/2013 is an antigenically distinct strain and should not be labeled as antigenically identical to the current A/California/07/2009 vaccine strain. This is important because vaccine preparations including Cal X-179A as the H1N1 component could produce different immunogenic responses than those vaccines including S.A. X-243 as the H1N1 component. Middle-aged adults would likely benefit more from receiving a vaccine with the more recent S.A. X-243 vaccine strain because it does not include an intact K166 HA-epitope and thus would presumably not elicit the K166 HA-specific Ab response that leaves them more susceptible to infection with current pH1N1 strains.

DISCUSSION

Our studies show that pH1N1 viruses that circulated during the 2013-2014 influenza season acquired a significant antigenic mutation that prevents binding of Abs elicited in a large number of middle-aged humans. For this reason, we propose that the pH1N1 vaccine strain should be updated. Conventional serological techniques used by most surveillance laboratories failed to recognize the K166Q HA mutation as

antigenically important (Arriola et al., 2014). HAI assays are based on serial sera dilutions and can only detect large antigenic changes. Many surveillance-based laboratories ignore twofold reductions in HAI titer because these laboratories typically process thousands of samples, which prohibit the experimental precision that is required to reliably detect twofold differences in these assays. However, a true twofold reduction in HAI titer against a mutated strain indicates an extremely immunodominant Ab response. Although we reproducibly detected as low as twofold HAI differences using K166Q HA mutated viruses (Tables 9 and 10 show results from three independent HAI experiments), our HAI assays likely underestimate the number of individuals that possess K166 HA-specific Abs. For example, we were able to isolate K166 HA-specific mAbs from pH1N1-vaccinated individuals whose sera yielded similar HAI titers using WT and K166Q mutated pH1N1 viruses. We also identified many human sera samples that had >fourfold reductions in HAI titer using pH1N1 viruses with the K166Q HA mutation, and it is worth noting that these results would likely have been missed if we pooled human sera samples or simply compared overall geometric means of HAI data with mutant viruses.

We attempted to recapitulate K166 HA-specific immunity in ferrets by sequentially infecting with sH1N1 strains and the A/California/07/2009 pH1N1 strain. Only three of eight ferrets sequentially infected with A/Chile/01/1983 and A/California/07/2009 mounted levels of K166 HA-specific Abs that could be detected by HAI assays. Outbred ferrets were used in these experiments, and the overall percentage of ferrets with K166 HA-specificity (Fig. 13) is similar to the overall percentage of humans born in the 1970s with K166 HA-specificity (Fig. 8A). We speculate that

variation in K166 HA-specificity in humans is due to variations in pre-exposure histories and genetic differences that impact B-cell repertoires.

Our results offer a possible antigenic explanation for the increased disease burden observed in middle-aged adults during the 2013–2014 influenza season. Given that the specificity of Ab responses is altered by pre-exposure history, we propose that conventional serological techniques used to identify antigenically novel viruses should be reevaluated. The usefulness of arbitrary HAI titer cutoffs and dependence on antisera generated in previously naïve ferrets (Koel et al., 2013; Smith et al., 2004) should be reconsidered. Although we believe that the pH1N1 vaccine should be updated immediately to a more recent strain (one that possesses HA Q166, similar to A/South Africa/3626/2013), it is not clear if a pH1N1 vaccine strain with Q166 HA will be able to break the original antigenic sin that currently exists in some middle-aged individuals. Further studies should be designed to determine if an updated H1N1 vaccine strain with Q166 HA elicits more effective Ab responses in different aged humans with distinct sH1N1 exposure histories.

MATERIALS AND METHODS

Human Donors

Studies involving human adults were approved by the Institutional Review Boards of Emory University, Vaccine and Gene Therapy Institute of Florida, the National Institute of Respiratory Diseases of Mexico, and the Wistar Institute. Informed consent was obtained. For all experiments, HAI and *in vitro* neutralization assays were completed at the Wistar Institute using preexisting and de-identified sera. We analyzed several sera

panels in this study. We analyzed sera from healthy donors collected at the New York Blood Center in February of 2014. We analyzed sera from healthy donors collected at the Center for Research in Infectious Diseases at the National Institute of Respiratory Diseases in Mexico. We analyzed sera and mAbs derived from healthy donors vaccinated with a monovalent pH1N1 vaccine in 2009 as previously described (Li et al., 2012). We analyzed sera collected from middle-aged adults (born 1977-1985) vaccinated with A/California/7/2009 at the Vaccine and Gene Therapy Institute of Florida (VGTI) during the 2013-2014 influenza season.

Viruses

Viruses possessing WT pH1N1 HA or K166Q pH1N1 HA were generated via reverse-genetics using HA and NA genes from A/California/07/2009 and internal genes from PR8. All of these viruses were engineered to possess the antigenically neutral D225G HA mutation (Chen et al., 2010a), which facilitates viral growth in fertilized chicken eggs. Viruses were grown in fertilized chicken eggs and the HA genes of each virus stock were sequenced to verify that additional mutations did not arise during propagation. sH1N1 strains (A/USSR/90/1977, A/Chile/01/1983, A/Singapore/06/1986, A/Texas/36/1991, A/New Caledonia/20/1999, and A/Solomon Islands/03/2006) were also grown in fertilized chicken eggs. We isolated a pH1N1 virus from respiratory secretions obtained from a patient from the Children's Hospital of Philadelphia in 2013 (named A/CHOP/1/2013 in this report). For this process, de-identified clinical material from the Children's Hospital of Philadelphia Clinical Virology Laboratory was added to Madin-Darby canine kidney (MDCK) cells (originally obtained from the National

Institutes of Health) in serum-free media with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin, HEPES, and gentamicin. Virus was isolated from the MDCK-infected cells 3 d later. We extracted viral RNA and sequenced the HA gene of A/CHOP/1/2013. We also used reverse-genetics to introduce glycosylation sites into A/California/07/2009 (pH1N1) HA. The consensus sequence for N-linked glycosylation (N-x-S/T) was added at HA residues 129, 131, and 163 by making the mutations D131T, D131N and N133T, and K163N, respectively. Similar results were obtained in HAI assays when we used glycosylation mutants grown in eggs or MDCK cells. Glycosylation at HA residues 129 and 131 was confirmed by treating concentrated virus with PNGase-F (New England Biolabs) under denaturing conditions. The CM1-4 anti-HA1 antibody was used as a primary antibody, and a donkey anti-mouse fluorescent secondary antibody (Licor) was used. Blots were imaged using the Licor Odyssey imaging system at 800nm (secondary antibody) and 700 nm (molecular weight marker).

Candidate Vaccine Strains

The HA genes of A/California/7/2009 WT and A/CHOP/1/2013 WT were cloned into the reverse genetics vector pHW2000. Site-directed mutagenesis was then used to generate both the A/California/7/2009 X-179A (Cal X-179A) and A/South Africa/3626/2013 X-243 (S.A. X-243) egg-adapted vaccine strain HA gene sequences. K212T and Q226R were inserted into the A/California/7/2009 WT HA to generate Cal X-179A HA. Likewise, D131E and L697V were inserted into the A/CHOP/1/2013 WT HA to generate S.A. X-243 HA. Reverse genetics were then used to rescue virus by transfecting a co-culture of 293T and MDCK cells with the Cal X-179A or S.A. X-243

HA genes along with the neuraminidase (NA) gene from A/California/07/2009 and the six internal influenza genes from PR8. Rescued virus was expanded in eggs one time and then sequence confirmed.

Animal Experiments

Murine experiments were performed at the Wistar Institute according to protocols approved by the Wistar Institute Institutional Animal Care and Use Committee. BALB/c mice (Charles River Laboratories) were injected with 25 µg of mAb intraperitoneally and then infected intranasally with 20,000 $TCID_{50}$ of WT or K166Q-HA pH1N1 virus 12 h later. As controls, some mice received an intraperitoneal injection of PBS before infection. Weight loss and survival was recorded for 11 days. Severely sick mice were euthanized. Ferret experiments were performed at VGTI in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories handbook. Fitch ferrets (Marshall Farms) were infected with 1×10^6 PFU of sH1N1 virus and bled 14 and 84 d later. These ferrets were then infected with the A/California/07/2009 pH1N1 strain and bled 14 d later. Some ferrets were sequentially infected with A/California/07/2009 (84 d between infections) and other ferrets were infected with A/California/07/2009 only once and bled 14 d later.

HAI Assays

Sera samples were pretreated with receptor-destroying enzyme (RDE, Key Scientific Products or Sigma-Aldrich) and HAI titrations were performed in 96-well

round-bottom plates (BD). Sera were serially diluted twofold and added to four agglutinating doses of virus in a total volume of 100 μL. Turkey red blood cells (RBCs, Lampire) were added [12.5 μL of a 2% (vol/vol) solution]. The RBCs were gently mixed with sera and virus and agglutination was read out after incubating for 60 min at room temperature. HAI titers were expressed as the inverse of the highest dilution that inhibited four agglutinating doses of turkey RBCs. Each HAI assay was performed independently on three different dates. Sera that had at least twofold reduced HAI titers using K166Q HA mutant viruses in three independent HAI assays were labeled as "K166 HA-specific." For the HAI assays to compare the vaccine strains, sera that had at least a twofold reduction in HAI titer against S.A. X-243 in three separate experiments were counted as specific for Cal X-179A.

ELISA Assays

Viruses for ELISAs were concentrated by centrifugation at 20,000 RPM for 1 h using a Thermo Scientific Sorvall WX Ultra 80 Centrifuge with a Beckman SW28 rotor. Concentrated viruses were then inactivated by beta-propiolactone (BPL; Sigma Aldrich) treatment. Viruses were incubated with 0.1% BPL and 0.1M HEPES (Cellgro) overnight at 4°C followed by a 90 min incubation at 37 °C. The 96-well Immulon 4HBX flat-bottom microtiter plates (Fisher Scientific) were coated with 20 HAU of BPL-treated virus per well overnight at 4°C. Each human mAb was serially diluted in PBS and added to the ELISA plates and allowed to incubate for 2 h at room temperature. As a control, we added the 70-1C04 stalk-specific mAb to verify equal coating of WT and K166Q HA virus. Next, peroxidase-conjugated goat anti-human IgG (Jackson Immunoresearch) was

incubated for 1 h at room temperature. Finally, SureBlue TMB Peroxidase Substrate (KPL) was added to each well and the reaction was stopped with addition of 250 mM HCl solution. Plates were extensively washed with distilled water between each ELISA step. Affinities were determined by nonlinear regression analysis of curves of six mAb dilutions (18 μg/mL to 74 ng/mL) using Graphpad Prism. mAbs were designated as K166 HA-specific if they had a K_d at least four times greater for the K166Q mutant than for the WT virus.

In Vitro Neutralization Assays

Sera were serially diluted twofold and then added to 100 $TCID_{50}$ units of virus and incubated at room temperature for 30 min. The virus-sera mixtures were then incubated with MDCK cells for 1 h at 37°C. Cells were washed and then serum-free media with TPCK-treated trypsin, HEPES, and gentamicin was added. Endpoints were determined visually 3 d later. Data are expressed as the inverse of the highest dilution that caused neutralization. All samples were repeated in quadruplicate and geometric mean titer is reported.

Receptor Binding Avidity Assays

Turkey RBCs (Lampire) were pretreated with various concentrations of α2-3,6,8 neuraminidase (NEB) for 60 min at 37°C. The treated RBCs were washed two times with PBS and added (as 1% v/v solutions) to eight agglutinating does of each virus (determined using non-treated turkey RBCs). Hemagglutination was measured after 60 min and receptor binding avidities were reported as the maximum concentration of

neuraminidase (units/mL) that still allows for full hemagglutination. Assay was completed three independent times.

Structural Modeling of HA Glycosylation Sites

Glycans were modeled onto positions 129 and 131 in the A/Solomon Islands/03/2006 HA crystal structure (PDB entry 3SM5) using the GLYCAM Web Glycoprotein Builder (www.glycam.org). The particular glycan used for modeling was an N-linked glycan with a trimannosyl core (DManpa1-6[DGlcpNAcb1-2DManpa1-3]DManpb1-4DGlcpNAcb1- 4DGlcpNAcb1-OH in Glycam notation), and default rotamer settings were used for modeling. To model the 131-glycosylation site, a T131N mutation was introduced using the PyMol structure viewer before the structure was uploaded to the GLYCAM-Web server.

Computational and Phylogenetic Analyses of HA Sequences

The occurrence of different amino acid identities at HA residues 166, 156, 157, and 158 (H3 numbering) was analyzed by downloading all full-length human pH1N1 sequences present in the Influenza Virus Resource (Bao et al., 2008) as of February 23, 2014. After purging sequences that were less than full-length, contained ambiguous nucleotide identities, lacked full (year, month, day) isolation dates, or were otherwise anomalous, the sequences were aligned. Each calendar year was broken into four equal partitions beginning with January 1, and the frequencies of different amino acids at each residue of interest for each partition was calculated and plotted. Only amino acids that reached a frequency of at least 1% in at least one of the year partitions are labeled in the

legend to the plot. For construction of phylogenetic trees, the sequence set was randomly subsampled to 10 sequences per quarter-year partition. BEAST (Drummond et al., 2012) was then used to sample from the posterior distribution of phylogenetic trees with reconstructed sequences at the nodes, after date stamping the sequences, using a Jones, Taylor, and Thornton (JTT) matrix (Jones et al., 1992) with a single rate category with an exponential prior, a strict molecular clock, and relatively uninformative coalescent-based prior over the tree. Fig. 7C shows a maximum clade credibility summary of the posterior distribution with branches colored according to the reconstructed amino acid identity at HA residue 166 with the highest posterior probability at their descendent nodes. The tree was visually rendered using FigTree. The input data and computer code used for this analysis can be found on GitHub at github.com/jbloom/pdmH1N1_ HA_K166_mutations.

Statistical Analyses

For all serum experiments, we excluded samples that did not have positive pH1N1 HAI titers. All samples that were pH1N1 HA-WT HAI-negative were also pH1N1 HA-K166Q HAI-negative. Samples were allocated to specific groups based on age of donor. The year of birth of each sample was available during the experiment, but this information was not assessed until after each experiment was completed. Variance of raw HAI titers was similar between different age groups. Fisher's exact tests were completed using SAS v9.3 software. For the receptor binding avidity assay, mean and standard error were calculated. A paired, two-tailed t-test was performed using SAS v9.3 software to determine significance.

FIGURES AND TABLES

Figure 6

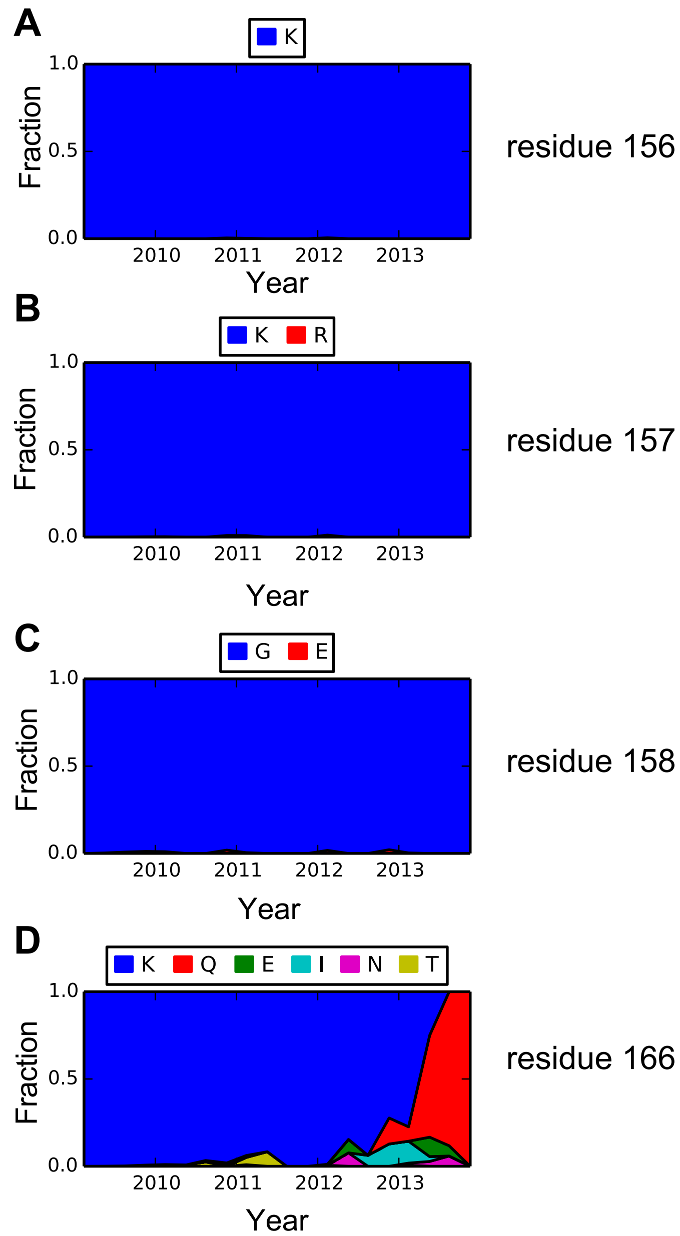


Figure 6. Sequence variation of pH1N1 HA.

The residues in the dominant antigenic site recognized by Abs elicited in previously naïve ferrets infected once with the A/California/07/2009 WT vaccine strain (HA

residues 156, 157, and 158 of the Sa antigenic site) are highly conserved in recent pH1N1 viruses (A–C). No variation greater than 1% has occurred at HA residue 156 and very little variation has occurred at HA residues 157 and 158. For comparison, HA residue 166 (D; also shown in Fig. 7B) underwent a complete change during the 2013-2014 influenza season.

Figure 7

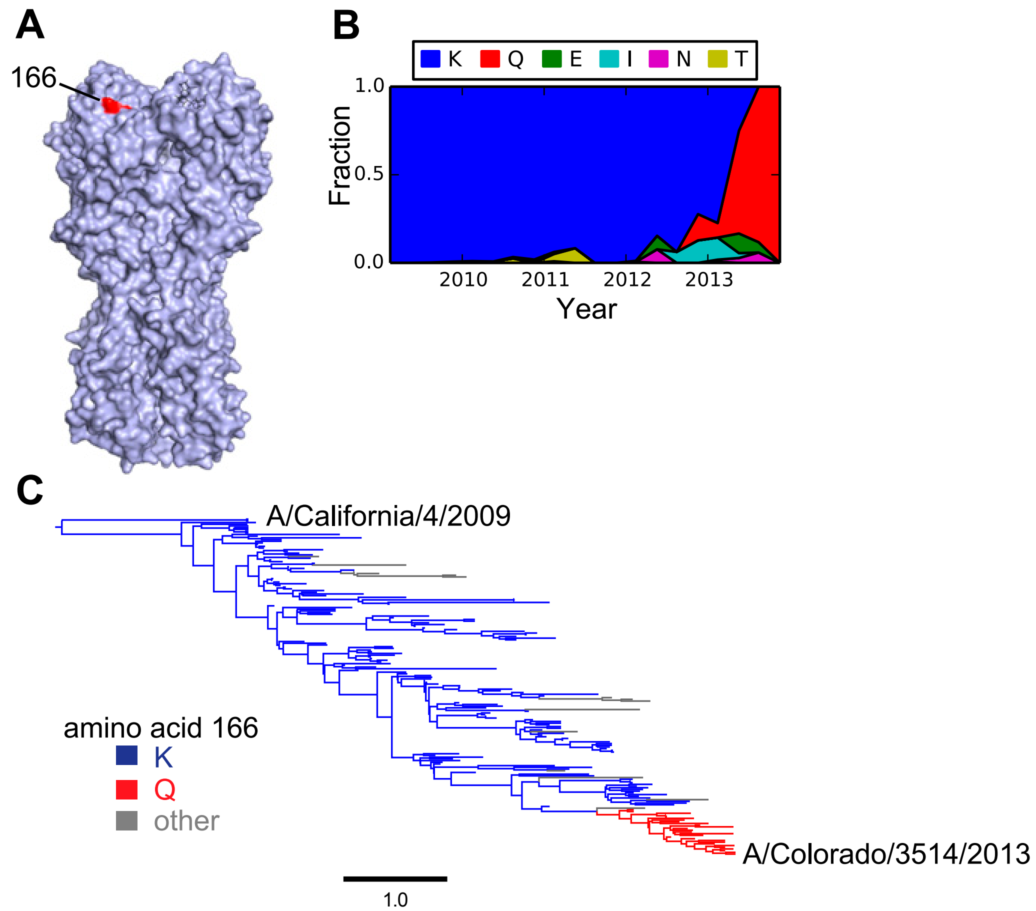
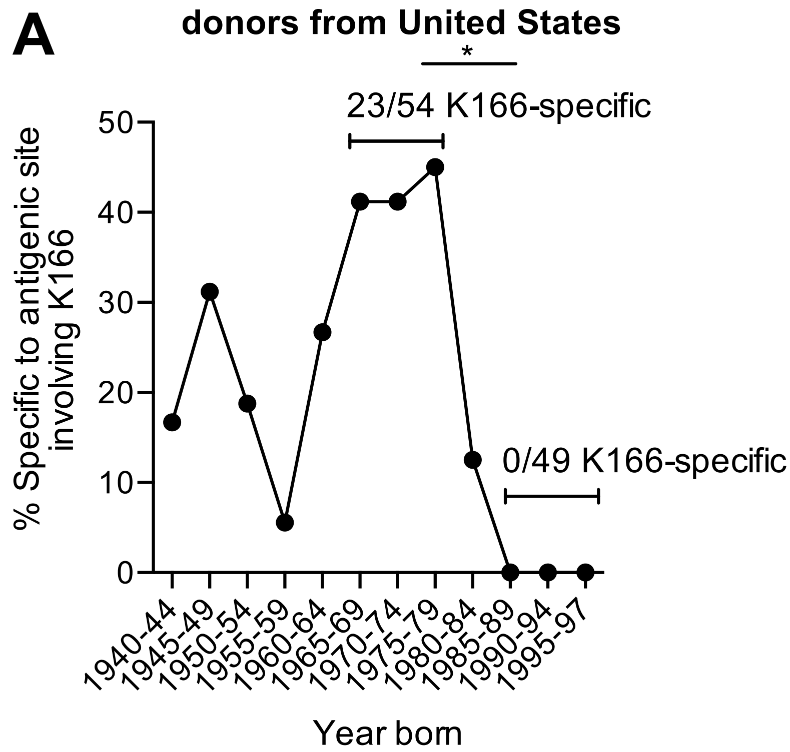
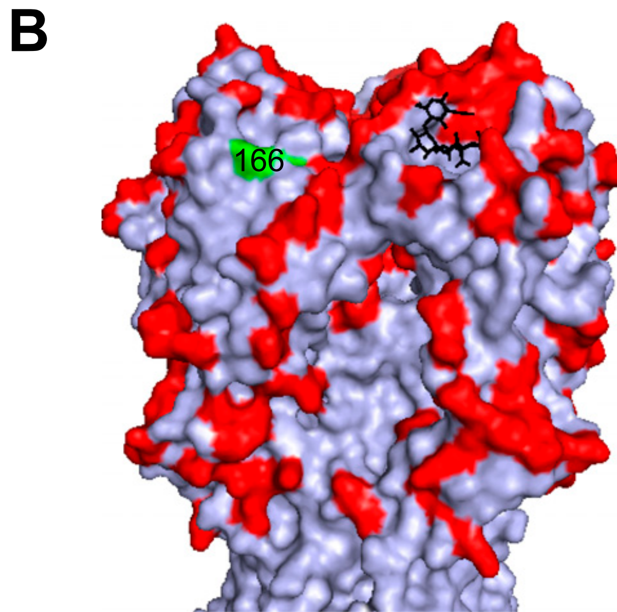


Figure 7. pH1N1 viruses rapidly acquired the K166Q HA mutation during the 2013-2014 influenza season.

(A) Residue K166 (red) is shown on the A/California/04/2009 HA trimer [PDB entry 3UBN (Xu et al., 2010)]. (B) Plotted is the frequency of different amino acid identities at HA residue 166 in pH1N1 HA sequences as a function of time. Nearly all pH1N1 isolates possessed K166 from 2009 to mid-2012, but most isolates possessed Q166 by the 2013–2014 season. (C) A phylogenetic tree of pH1N1 viruses with branches colored according to amino acid identity at residue 166 illustrates the rapid fixation of K166Q in pH1N1 isolates that circulated in 2013.

Figure 8





Homology between A/Chile/01/1983 and A/California/07/2009

Figure 8. Middle-aged adults possess Abs that bind to a region of HA that became mutated in pH1N1 viruses during the 2013-2014 influenza season.

(A) Sera were isolated from healthy donors (n = 195) from the state of New York during the 2013–2014 influenza season. HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each sera sample, we completed three independent HAI assays. Raw HAI data are reported in Table 9. Percentages of samples that had at least a twofold reduction in HAI titer against the mutant virus in three independent experiments are shown. K166 HA-specificity of sera from individuals born between 1965 and 1979 is statistically significant compared with K166 HA-specificity of sera from individuals born after 1985 (Fisher's exact test; $^{*}P < 0.0001$). (B) Homology between the A/Chile/01/1983 sH1 and the A/California/04/2009 pH1 are shown using the crystal structure of the A/California/04/2009 HA [PDB entry 3UBN (Xu et al., 2010)]. Residue K166 is colored green. Amino acids that differ between A/Chile/01/1983 and A/California/04/2009 are shown in red. The glycan receptor is shown in black.

Figure 9.

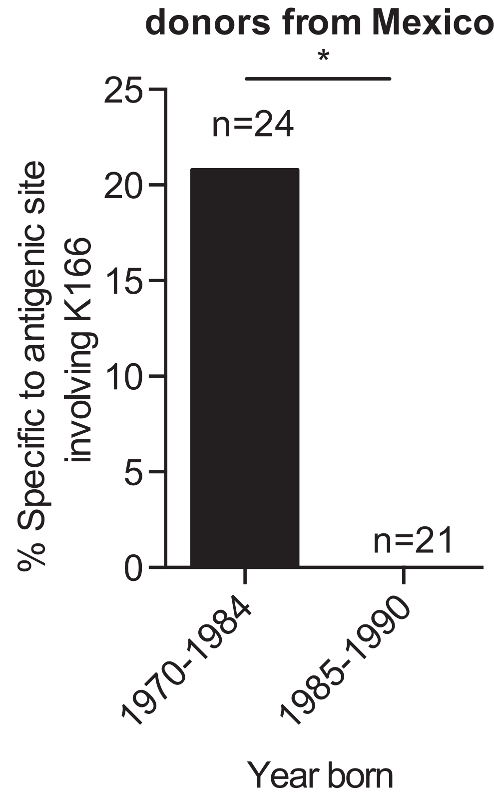


Figure 9. Mexican donors born before 1985 possess Abs that bind to the region of HA that became mutated in pH1N1 viruses during the 2013-2014 influenza season. Sera were isolated from healthy donors (n = 45) at the Center for Research in Infectious Diseases at the National Institute of Respiratory Diseases in Mexico during the 2013–2014 influenza season. HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each sera sample, we completed three independent HAI assays. Raw HAI data are reported in Table 10. Percentages of samples that had at least a twofold reduction in HAI titer using the mutant virus in three independent experiments are shown. (Fisher's exact test; *P < 0.05).

Figure 10

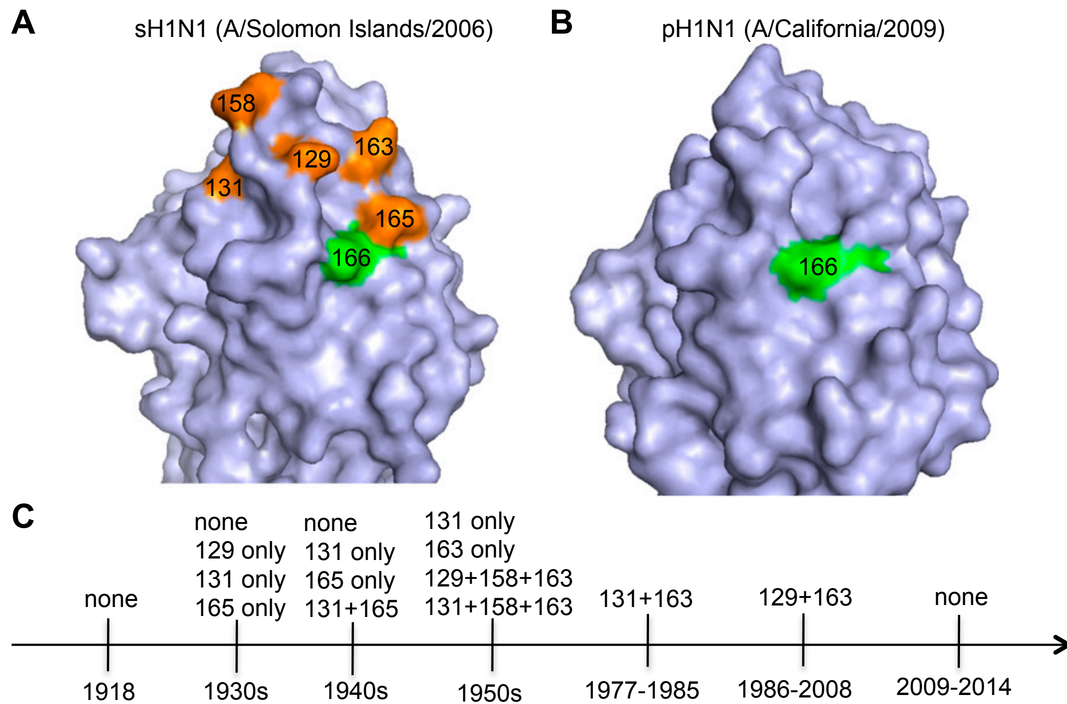
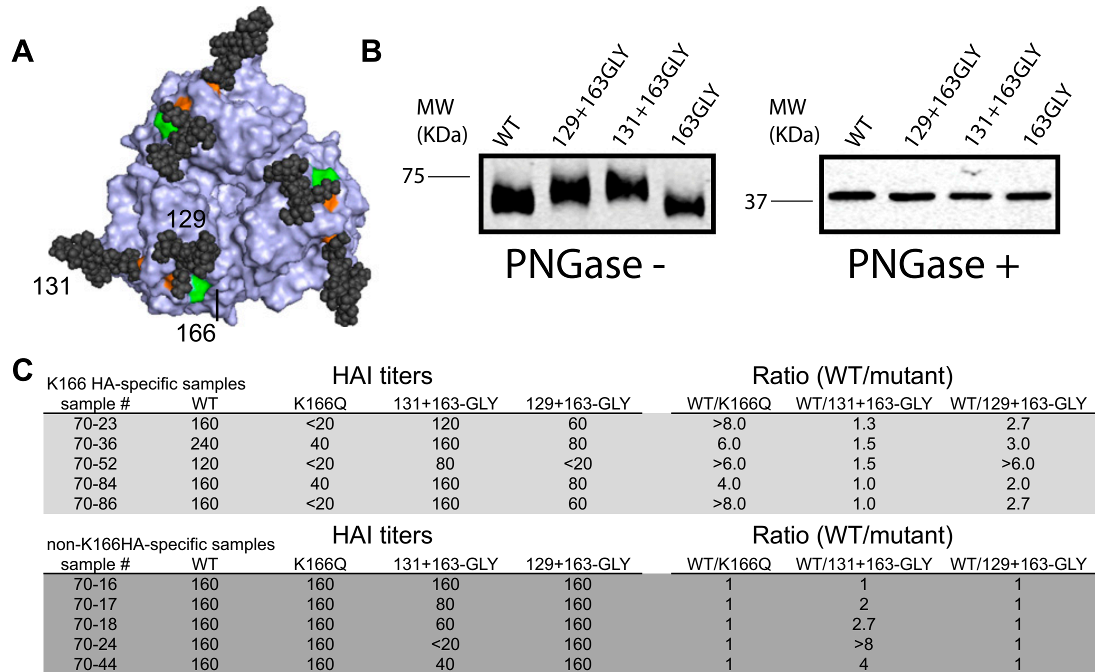


Figure 10. Glycosylation status of various H1N1 viruses.

The crystal structures of sH1N1 (A) and pH1N1 (B) HAs are shown (PDB entries 3SM5 and 3UBN). Glycosylation sites that have appeared from 1918 to 2008 in sH1N1 viruses are highlighted in orange and residue 166 is shown in green. (C) Glycosylation status of H1N1 viruses circulating during different time periods is shown as reported previously (Wei et al., 2010). Very few viral sequences are available for 1930–1950 viruses and variability of glycosylation sites in these viruses likely relates to egg adaptations. The majority of sH1N1 viruses circulating between 1977 and 1985 have the 131- and 163-glycosylation sites and the majority of sH1N1 viruses circulating between 1986 and 2008 have the 129- and 163-glycosylation sites. Of note, although HA residues 129 and 131

are very close in the linear sequence, they are located on opposite sides of the Sa/Sb ridge. The 131-glycosylation site is not expected to cover the K166 HA-epitope, whereas the 129-glycosylation site potentially shields the K166 HA-epitope.

Figure 11



K166 HA-specific samples sample #	HAI titers WT	K166Q	131+163-GLY	129+163-GLY	Ratio (WT/mutant) WT/K166Q	WT/131+163-GLY	WT/129+163-GLY
70-23	160	<20	120	60	>8.0	1.3	2.7
70-36	240	40	160	80	6.0	1.5	3.0
70-52	120	<20	80	<20	>6.0	1.5	>6.0
70-84	160	40	160	80	4.0	1.0	2.0
70-86	160	<20	160	60	>8.0	1.0	2.7

non-K166HA-specific samples sample #	HAI titers WT	K166Q	131+163-GLY	129+163-GLY	Ratio (WT/mutant) WT/K166Q	WT/131+163-GLY	WT/129+163-GLY
70-16	160	160	160	160	1	1	1
70-17	160	160	80	160	1	2	1
70-18	160	160	60	160	1	2.7	1
70-24	160	160	<20	160	1	>8	1
70-44	160	160	40	160	1	4	1

Figure 11. Modeling glycosylation sites at HA residues 129 and 131.

(A) Modeling of the putative glycosylation sites at residues 129 and 131 on the HA of A/Solomon Islands/6/ 2006 (PDB entry 3SM5) was completed using Glycam software. HA residues 129 and 131 are shown in orange and predicted sugars are shown in black. HA residue 166 is shown in green. The 129-glycosylation site present in most sH1N1 isolates circulating after 1985 is predicted to shield the antigenic epitope involving HA residue 166. The 131-glycosylation site is not predicted to shield the antigenic epitope involving HA residue 166. (B) Viruses possessing A/California/07/2009 HA with different putative glycosylation sites were created by reverse-genetics. HA from viruses

with putative glycosylation sites introduced at HA residues 129+163 and 131+163 migrated slower compared with unmodified HA and HA from viruses with a putative glycosylation site at only HA residue 163. This finding indicates that HA residues 129 and 131, but not HA residue 163, were glycosylated in the reverse-genetics derived viruses. HA from all viruses migrates similarly following PNGase treatment. PNGase treatment was completed under reducing conditions, so HA migrated faster compared with no PNGase treatment. (C) HAI assays were completed using the reverse-genetics derived viruses possessing A/California/07/2009 with different glycosylation sites. Five K166 HA-specific samples and five non-K166 HA-specific human samples were tested. Data are representative of three independent experiments.

Figure 12.

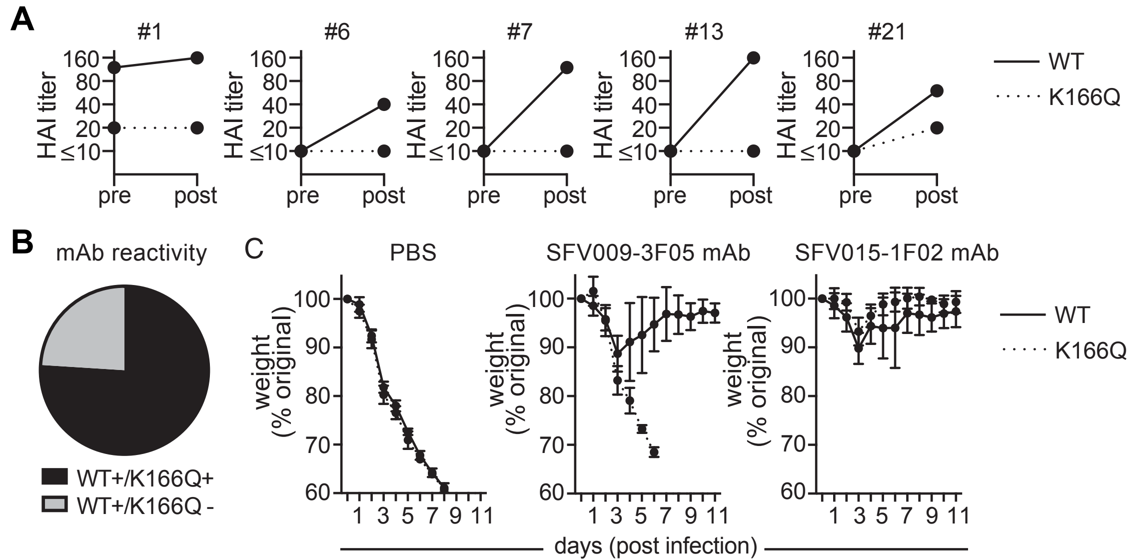


Figure 12. Vaccination of middle-aged adults with the current pH1N1 vaccine strain elicits Abs that bind to a region of HA that is now mutated in most pH1N1 isolates. (A) Healthy adult volunteers were vaccinated with a monovalent pH1N1 vaccine in 2009. Sera were isolated pre-vaccination and 30 d post-vaccination and HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. Shown are HAI titers for donors that possessed K166 HA-specific Abs following vaccination. Data are representative of three independent experiments. Raw HAI titers for all donors are shown in Table 12. (B) ELISAs were completed using mAbs isolated from healthy adult volunteers that were vaccinated with a monovalent pH1N1 vaccine in 2009. ELISAs were coated either with WT A/California/07/2009 or A/California/07/2009 with a K166Q HA mutation. Shown are percentage of mAbs that bound to both viruses and percentage of mAbs that bound to

the WT virus but not the K166Q mutant virus (n = 42 mAbs). Data are representative of two independent experiments. (C) A K166 HA-specific mAb (SFV009-3F05) or a mAb that recognizes both WT and K166Q-HA pH1N1 (SFV015-1F02) were injected into BALB/c mice (n = 4 per group). Twelve hours later, mice were then infected with 20,000 $TCID_{50}$ of WT or K166Q-HA virus and weight loss and survival were recorded for 11 d. Data are representative of two independent experiments.

Figure 13

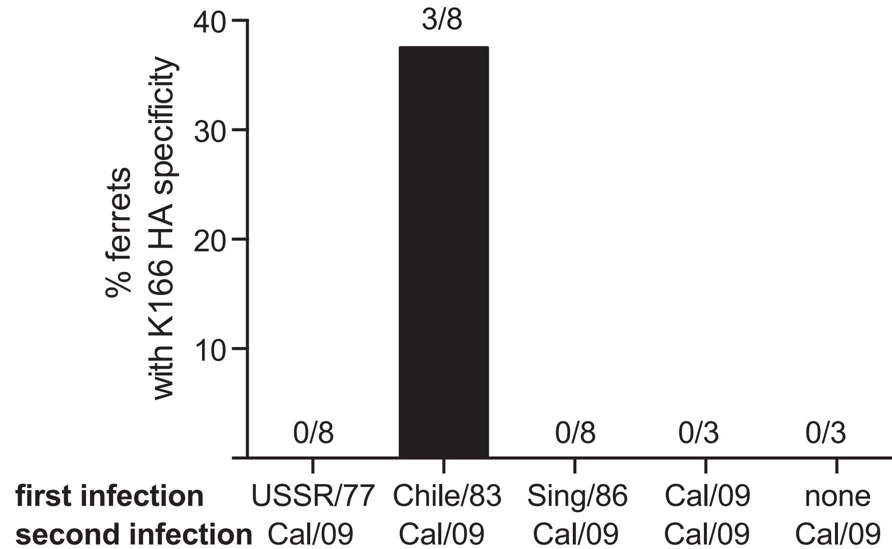


Figure 13. Ferrets sequentially infected with A/Chile/01/1983 and A/California/07/2009 develop K166 HA-specific Abs.

Ferrets were infected with a sH1N1 virus and then re-infected 84 d later with the A/California/07/2009 pH1N1 virus. Sera were collected 14 d after the second infection and HAI assays were completed using WT and K166Q-HA pH1N1 viruses. Shown are percentages of samples that had at least a twofold reduction in HAI titer using the K166Q HA mutant virus in three independent experiments. Raw HAI titers are shown in Table 13. The difference in K166 HA-specificity is statistically significant comparing the A/Chile/01/1983-A/California/07/2009 group with the rest of the groups (3 of 8 vs. 0 of 22; Fisher's exact test $P < 0.05$).

Figure 14

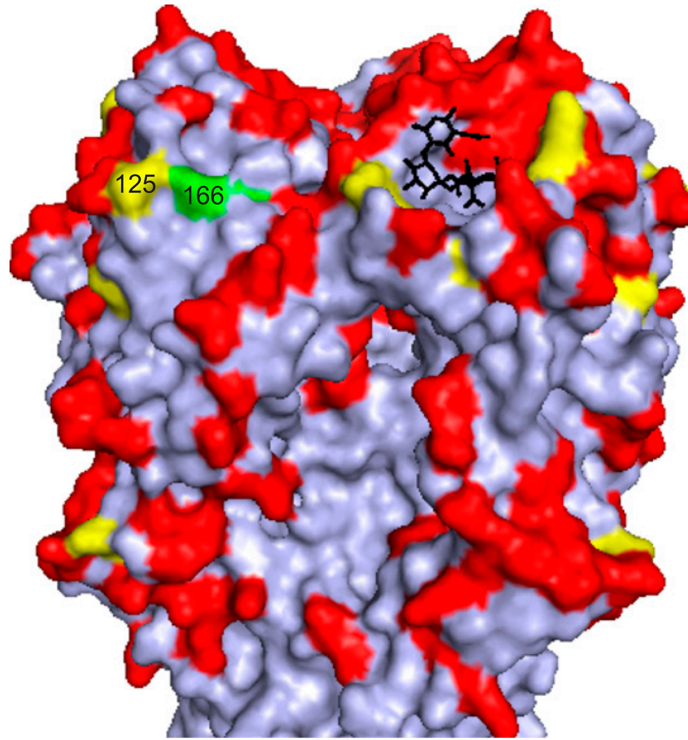


Figure 14. Homology between A/USSR/90/1977, A/Chile/01/1983, and A/California/07/2009.

HA residues that differ between A/Chile/01/1983 and A/California/07/2009 are shown in red. A few additional HA residues differ between the HAs of A/USSR/90/1977 and A/California/07/2009, and these are colored yellow. Of note, A/Chile/01/1983 and A/California/07/2009 both possess S125 whereas A/USSR/90/1977 possess R125. HA residues 125 and 166 are adjacent to each other in the structure. PDB entry 3UBN (A/California/04/2009 HA) was used to make this figure.

Figure 15

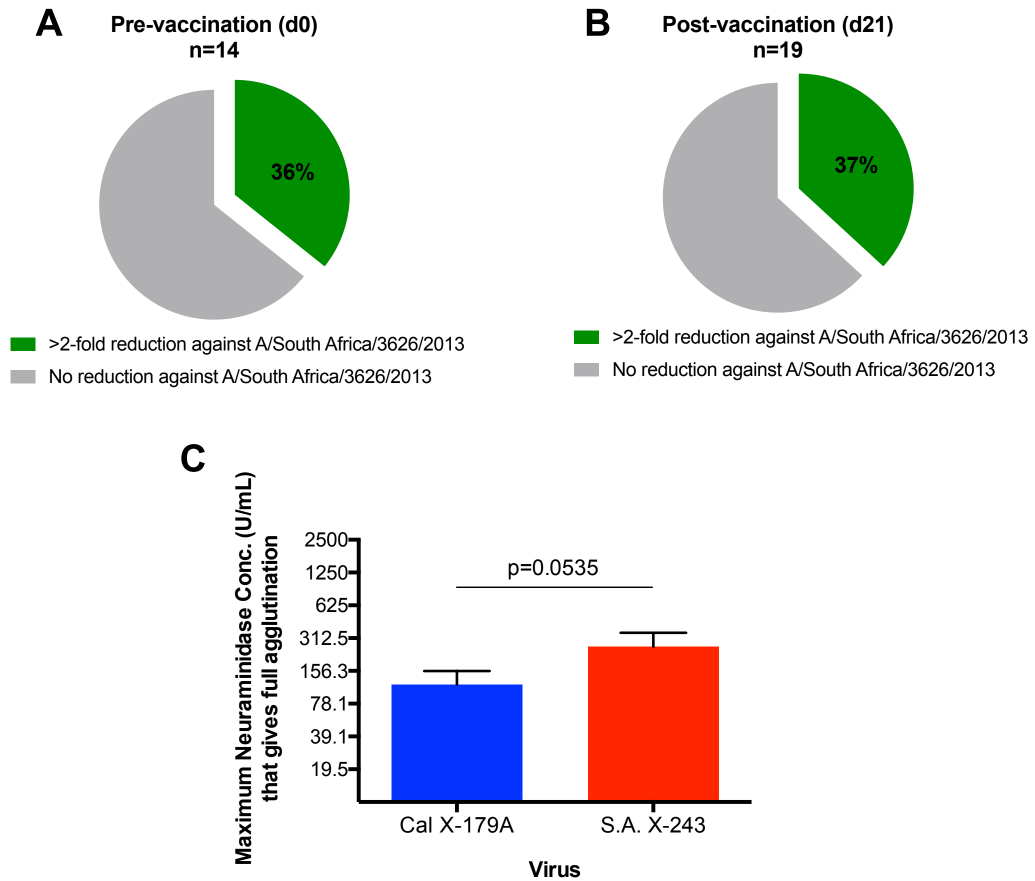


Figure 15. Vaccinated human sera demonstrate the new candidate H1N1 vaccine strain, A/South Africa/3626/2013, is antigenically distinct from A/California/07/2009.

Sera were collected from vaccinated middle-aged adults (born 1977-1985) during the 2013-2014 influenza season at the Vaccine and Gene Therapy Institute of Florida (n = 20). Both (A) pre- and (B) post-vaccination HAI titers were determined against the Cal X-179A and S.A. X-243 vaccine strains. Displayed is the fraction of individuals who had titers against Cal X-179A and at least a twofold decrease in HAI titer against S.A. X-243

in three independent experiments. (C) Relative binding avidity between the two viruses was determined by pre-treating turkey RBCs with different dilutions of α2-3,6,8 neuraminidase before measuring hemagglutination. Neuraminidase concentration is reported as the maximum units/mL of neuraminidase that still allows for full hemagglutination. Mean and standard error bars are shown. The difference in receptor binding avidity between Cal X-179A and S.A. X-243 is insignificant following three independent assays ($p=0.0535$, paired two-tailed t-test).

Table 9

Sample ID	YOB	Exp. 1			Exp. 2			Exp. 3		
		HAI titers		Ratio	HAI titers		Ratio	HAI titers		Ratio
		WT	K166Q	WT/K166Q	WT	K166Q	WT/K166Q	WT	K166Q	WT/K166Q
40–60	1940	160	80	2.00	160	80	2.00	160	60	2.67
40–49	1943	80	<20	>4.00	60	<20	>3.00	80	<20	>4.00
40–05	1948	160	80	2.00	160	80	2.00	160	80	2.00
40–02	1948	120	60	2.00	60	30	2.00	80	30	2.67
40–44	1948	320	80	4.00	320	80	4.00	320	80	4.00
40–14	1949	240	80	3.00	240	80	3.00	240	80	3.00
40–16	1949	240	<20	>12.00	160	<20	>8.00	160	<20	>8.00
50–55	1952	160	80	2.00	160	60	2.67	160	80	2.00
50–11	1952	120	40	3.00	80	30	2.67	80	30	2.67
50–43	1953	80	40	2.00	80	40	2.00	80	40	2.00
50–06	1959	120	60	2.00	80	40	2.00	80	30	2.67
60–04	1963	80	40	2.00	80	40	2.00	80	40	2.00
60–10	1963	160	80	2.00	240	80	3.00	160	60	2.67
60–13	1964	240	120	2.00	320	120	2.67	240	120	2.00
60–25	1964	60	20	3.00	80	30	2.67	60	20	3.00
60–14	1967	160	80	2.00	120	60	2.00	120	60	2.00
60–23	1967	320	120	2.67	320	160	2.00	320	160	2.00
60–44	1968	160	80	2.00	160	80	2.00	160	80	2.00
60–28	1969	320	160	2.00	320	160	2.00	320	160	2.00
60–41	1969	120	60	2.00	160	80	2.00	160	80	2.00
60–63	1969	120	60	2.00	120	60	2.00	120	60	2.00
60–35	1969	80	40	2.00	80	30	2.67	80	30	2.67
70–56	1971	80	40	2.00	80	30	2.67	80	40	2.00
70–52	1971	160	<20	>8.00	120	<20	>6.00	80	<20	>4.00
70–60	1972	160	80	2.00	160	80	2.00	160	80	2.00
70–74	1972	160	80	2.00	240	80	3.00	240	80	3.00
70–36	1972	240	60	4.00	240	30	8.00	320	30	10.67
70–84	1973	120	40	3.00	160	30	5.33	160	40	4.00
70–86	1973	160	40	4.00	160	30	5.33	160	40	4.00
70–13	1975	640	320	2.00	640	320	2.00	640	320	2.00
70–54	1975	160	80	2.00	80	30	2.67	120	60	2.00
70–25	1975	160	80	2.00	80	30	2.67	120	40	3.00
70–10	1975	160	30	5.33	80	30	2.67	80	30	2.67
70–23	1975	160	<20	>8.00	120	<20	>6.00	160	<20	>8.00
70–82	1976	160	80	2.00	160	60	2.67	160	80	2.00
70–50	1976	240	80	3.00	160	60	2.67	120	60	2.00
70–80	1978	320	160	2.00	320	160	2.00	320	160	2.00
70–19	1978	80	<20	>4.00	80	<20	>4.00	80	<20	>4.00
80–16	1980	80	30	2.67	60	20	3.00	80	40	2.00
80–48	1983	640	120	5.33	640	80	8.00	640	80	8.00

Table 9. HAI titers using sera collected from healthy human donors from the United States.

Sera were isolated from 195 healthy donors from the state of New York during the 2013-2014 influenza season. HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For

each sera sample, we completed three independent HAI assays. Shown are samples that had at least a twofold reduction in HAI titer using the K166Q mutant virus in all three independent experiments. YOB, year of birth.

Table 10

		Exp. 1			Exp. 2			Exp. 3		
		HAI titers		Ratio	HAI titers		Ratio	HAI titers		Ratio
Sample ID	YOB	WT	K166Q	WT/K166Q	WT	K166Q	WT/K166Q	WT	K166Q	WT/K166Q
N-33	1973	160	60	2.67	120	40	3.00	80	30	2.67
N-42	1975	40	<10	>4.00	30	<10	>3.00	30	<10	>3.00
N-93	1977	120	30	4.00	60	30	2.00	60	20	3.00
N-72	1979	30	<10	>3.00	30	<10	>3.00	20	<10	>2.00
N-70	1980	160	80	2.00	120	60	2.00	120	40	3.00

Table 10. HAI titers using sera collected from healthy human donors from Mexico. Sera were isolated from 45 healthy donors from Mexico during the 2013-2014 influenza season. HAI assays were performed using viruses with either WT A/California/07/2009 HA or A/California/07/2009 HA with a K166Q HA mutation. For each sera sample, we completed three independent HAI assays. Shown are samples that had at least a twofold reduction in HAI titer using the K166Q mutant virus in all three independent experiments.

Table 11

Sample ID	YOB	HAI titers: A/CAL/07/09-WT HA	HAI titers: A/CHOP/1/2013	In vitro neutralization titers: A/CAL/07/09-WT HA	In vitro neutralization titers: A/CAL/07/09-K166Q HA
40–49	1943	20	<10	24	12
40–44	1948	160	30	453	95
40–16	1949	80	<10	160	20
70–52	1971	40	<10	190	28
70–36	1972	120	10	381	95
70–84	1973	40	<10	160	57
70–86	1973	60	20	190	67
70–23	1975	80	<10	226	20
70–19	1978	30	<10	95	10
80–48	1983	320	30	1,076	80
80–10	1986	160	160	761	1,810
80–26	1986	80	80	113	160
80–74	1986	40	40	135	113
80–30	1987	20	10	67	113
80–34	1987	160	160	381	538
80–56	1988	20	<10	17	34
90–01	1990	40	10	48	80
90–24	1991	60	20	34	95
90–12	1992	160	60	190	226
90–13	1992	80	80	190	320

Table 11. Characterization of K166 HA-specific human sera.

We further characterized several sera samples that had immunodominant K166 HA-specific Abs (light gray: K166 HA-specific sera from individuals born before 1985; darker gray: sera from individuals born after 1985). HAI assays were completed using viruses with A/California/07/2009 HA-WT and a primary pH1N1 virus isolated from the Children's Hospital of Philadelphia (CHOP) in 2013 (A/CHOP/1/2013) that contains Q166. Additionally, *in vitro* neutralization assays were completed using the reverse-genetics derived viruses. Data are representative of two independent experiments. For each neutralization assay, each sample was titered four times, and the geometric mean of these quadruplicate samples is reported.

Table 12

		Visit 1 (prevaccine)							Visit 2 (postvaccine)	
ID	YOB	USSR/77	Chile/83	Texas/91	NC/99	SI/06	CAL/09	CAL/09-K166Q	CAL/09-WT	CAL/09-K166Q
K166-specific donors										
1	1979	240	160	320	60	80	120	20	160	20
6	1966	160	120	240	120	240	<10	<10	40	10
7	1966	40	60	30	10	60	<10	<10	120	<10
13	1982	80	40	80	40	60	10	<10	160	10
21	1958	60	30	80	40	80	<10	<10	60	20
Other donors										
2	1976	80	80	120	<10	<10	60	60	80	60
5	1949	10	<10	<10	10	40	10	<10	40	40
8	1961	40	<10	30	80	120	<10	<10	80	80
9	1977	80	80	80	<10	<10	<10	<10	120	80
10	1983	80	<10	320	240	320	<10	<10	240	320
15	1961	<10	<10	<10	<10	<10	<10	<10	80	80
17	1956	40	<10	30	80	120	10	<10	20	20
19	1961	80	40	160	80	80	<10	<10	20	20
20	1945	40	<10	<10	<10	<10	<10	<10	240	320
22	1956	30	30	60	<10	<10	<10	<10	240	240
23	1975	40	<10	240	80	120	<10	<10	160	160
24	1968	<10	<10	30	<10	<10	<10	<10	60	60

Table 12. Vaccination elicits K166 HA-specific Ab responses in humans.

HAI assays were completed using sera isolated from healthy donors before and after vaccination with the monovalent pH1N1 virus in 2009. Post-vaccine sera were collected 30 d after vaccination. HAI assays using post-vaccination sera were completed three independent times and assays using pre-vaccine sera were completed two independent times. Red indicates HAI titers equal to or less than 10 and yellow indicates HAI titers greater than 10.

Table 13

First infection	Second infection	Group	Animal no.	Day 14 WT	Day 14 K166Q	Day 84 WT	Day 84 K166Q	Day 98 (14 d after second infection) WT	Day 98 (14 d after second infection) K166Q
USSR/90/77	A/California/07/09	Group 1	7982	<10	<10	<10	<10	800	800
		Group 1	7983	<10	<10	<10	<10	800	800
		Group 1	7984	<10	<10	<10	<10	300	300
		Group 1	7985	<10	<10	<10	<10	800	600
		Group 1	7991	<10	<10	<10	<10	2,400	2,400
		Group 1	7993	<10	<10	<10	<10	800	1,200
		Group 1	7994	<10	<10	<10	<10	600	600
		Group 1	7995	<10	<10	<10	<10	400	600
A/Chile/01/83	A/California/07/09	Group 2	7987	<10	<10	<10	<10	1,200	800
		Group 2	7988	<10	<10	<10	<10	800	400
		Group 2	7989	<10	<10	<10	<10	800	400
		Group 2	7990	<10	<10	<10	<10	1,600	1,600
		Group 2	7996	<10	<10	<10	<10	1,200	400
		Group 2	7997	<10	<10	<10	<10	240	160
		Group 2	8000	<10	<10	<10	<10	600	800
		Group 2	8001	<10	<10	<10	<10	800	800
A/Singapore/06/86	A/California/07/09	Group 3	1487	<10	<10	80	120	6,400	6,400
		Group 3	1488	<10	<10	<10	<10	3,200	3,200
		Group 3	1481	<10	<10	<10	<10	2,400	3,200
		Group 3	1504	<10	<10	60	80	4,800	6,400
		Group 3	8002	<10	<10	<10	<10	1,600	2,400
		Group 3	8009	<10	<10	<10	<10	1,600	2,400
		Group 3	8010	<10	<10	<10	<10	4,800	6,400
		Group 3	8012	<10	<10	<10	<10	1,600	2,400
A/California/07/09	A/California/07/09	Group 4	8013	400	400	200	200	160	160
		Group 4	8014	800	800	400	400	2,400	2,400
		Group 4	8015	800	800	300	200	2,400	1,600
None	A/California/07/09	Group 5	1486	ND	ND	ND	ND	1,600	2,400
		Group 5	1497	ND	ND	ND	ND	1,600	1,600
		Group 5	1492	ND	ND	ND	ND	1,200	1,600

Table 13. Analyses of sera from ferrets sequentially infected with sH1N1 viruses and pH1N1.

Ferrets were infected with a sH1N1 virus and bled 14 d and 84 d later. Animals were then infected with a pH1N1 strain and bled 14 d after second infection (98 d post-first infection). Sera were isolated and HAI assays were completed using WT and K166Q-HA pH1N1 viruses. Data are representative of three independent HAI assays. The three ferrets with K166 HA-specific Ab responses had at least a twofold reduction in HAI titer against the K166Q mutant virus in three independent experiments. Red highlights twofold or greater reductions in HAI titer using K166Q-HA pH1N1 virus compared to WT-HA pH1N1 virus. ND, not determined.

CHAPTER 5 – OVERALL CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

In our studies, we explored multiple aspects of the current influenza virus surveillance and vaccine strain selection process. First, we found that recent H3N2 viruses rapidly acquire mutations when propagated in cell culture. Mutations arose in both HA and NA after a single passage of clinical isolates in MDCK cells. We identified NA mutations that resulted in NA-dependent sialic acid binding and an HA mutation that increased receptor binding avidity. Both HA and NA mutations increased viral infectivity and hemagglutination titers. We found that these mutations altered HAI assays that are commonly used for the antigenic characterization of influenza virus isolates. Second, we determined that a single mutation in HA antigenic site B of H3N2 viruses contributed to the influenza vaccine mismatch during the 2014-2015 season. Inclusion of the F159S HA mutation in HA antigenic site B drastically decreased the ability of ferret, sheep, and human Abs to bind and neutralize the virus. Because a single mutation in an antigenic site can result in a vaccine mismatch, accurate surveillance and correct antigenic characterization of circulating strains is vital in producing an effective influenza vaccine each year. Third, we examined how different sH1N1 pre-exposure histories can result in unique anti-pH1N1 Ab repertoires. Over 40% of middle-aged adult blood donors (born 1965-1979) were found to possess Abs specific for an HA epitope that recently acquired a mutation (K166Q). Vaccinated middle-aged adults also possessed Abs with this specificity, which left them more susceptible to influenza infection with pH1N1 strains containing Q166. Sera isolated from ferrets recovering from a primary pH1N1 infection

did not possess Abs that recognized this epitope, but sera from ferrets sequentially infected with a 1983 sH1N1 virus and then challenged with the 2009 pH1N1 virus did possess Abs that were sensitive to the K166Q mutation. Therefore, Abs in the sera of ferrets recovering from primary influenza virus infections do not have the same specificity of Abs isolated from humans who have been sequentially exposed to different influenza virus strains.

Mutations acquired during viral propagation complicate influenza surveillance

Cell culture-derived mutations have been shown to alter HA stability (Nakowitsch et al., 2014; Nakowitsch et al., 2011), reduce immunogenicity (Chen et al., 2010b), and affect NA activity (Wang et al., 2013). Over the past two decades, H3N2 influenza viruses have seen a steady decline in their receptor binding avidity (Gulati et al., 2013; Lin et al., 2012). This has led to poor growth during cell culture propagation because the viruses are unable to bind to their sialic acid receptors and infect cells for replication. We passaged 10 clinical isolates from H3N2-infected children on MDCK cells, and observed a high rate of mutations in both HA and NA. The P237L HA mutation increased receptor binding avidity, possibly by stabilizing the HA trimer because HA residue 237 is located at the trimer interface. The D151G and D151N NA mutations that arose were responsible for NA-dependent binding of sialic acid, as has been seen previously (Lin et al., 2010; Zhu et al., 2012). Both types of mutations are likely to effect antigenic analyses, as we showed specifically with the P237L HA reverse genetics virus in an HAI assay, and as other groups have shown with NA-dependent binding in HAI assays (Lin et al., 2010).

This rapid acquisition of mutations in HA and NA during viral passage presents a major problem for surveillance. For example, HA antigenic clade 3C.2a H3N2 viruses acquire mutations when grown in MDCK cell culture (Skowronski et al., 2016). When attempting to choose the correct viral strain to include in the influenza vaccine each year, it is important to isolate and characterize the viruses that are actually circulating. If isolates are acquiring mutations during propagation (before any analyses are completed), the sequencing data and antigenic characteristics actually represent the cell culture-adapted H3N2 viruses, not the naturally occurring H3N2 viruses of interest.

Now that cell culture-derived mutations have been established as a major complication to surveillance and vaccine selection, steps are being implemented to grow and sequence influenza viruses more reliably. Previously, surveillance has been done with a "passage-first" mentality where clinical isolates are immediately expanded in cell culture to obtain a workable stock, then antigenically characterized, and finally sequenced if an antigenic difference is detected (Krauss et al., 2012; Stöhr et al., 2012). Now, the CDC is working on shifting to a "sequence-first" approach to surveillance, where clinical isolates are immediately sequenced directly from the primary isolate before any propagation or antigenic analyses are completed (Dormitzer et al., 2013). This method eliminates the possibility that cell culture adaptive mutations will arise before sequencing can be completed. The other main change in surveillance is the discovery that using a different cell culture system can actually decrease the amount of mutations that arise during propagation. Instead of using the classic MDCK cells for viral propagation, using MDCK-SIAT1 cells for viral propagation allows recent H3N2 viruses to grow without the need for adaptive mutations (Abdoli et al., 2016; Krauss et al., 2012; Oh et

al., 2008). MDCK-SIAT1 cells are a form of MDCK cells that have been engineered to express a higher concentration of α2,6-linked sialic acids on their surface (Matrosovich et al., 2003), which serve as the receptor for human influenza viruses. With more receptor present, even the H3N2 viruses with very low receptor binding avidity are able to infect the cells and grow well. So by using a "sequence-first" approach and MDCK-SIAT1 cells for viral propagation, the previous complications seen in H3N2 surveillance due to cell culture-adaptive mutations should be avoided.

NA-dependent receptor binding is a recent observation in the influenza field

For as long as the influenza research field has existed, the central dogma is that HA is responsible for binding and entry, and NA is responsible for release. However, multiple groups have now begun to identify viruses that bind to sialic acid through NA instead of HA (Lin et al., 2010; Zhu et al., 2012). The caveat here is that, so far, NA-dependent binding has only been detected following propagation in cell culture. Similar to what we found in Chapter 2, other groups have concluded that primary isolates do not contain the D151G or D151N NA mutations responsible for the NA-binding phenotype (Lee et al., 2013; Okomo-Adhiambo et al., 2010). The question now is whether a continued reduction of HA receptor binding avidity in H3N2 viruses will result in the need for NA-dependent sialic acid binding even in naturally circulating viruses. Lin and colleagues demonstrated through reverse genetics that only a single mutation at NA residue 151 is required for NA-dependent binding. They inserted the D151G or D151N NA mutations and observed increased receptor binding via higher levels of NA-dependent agglutination (Lin et al., 2010). With only a single mutation required for this

NA-dependent binding phenotype, it seems possible that a circulating isolate with NA-dependent binding could emerge. However, mutations at D151 decrease NA sialidase activity and may hinder the virus without compensatory mutations (Zhu et al., 2012). Overall, NA-dependent binding of sialic acid is a major shift in the dogma of the influenza community. It is currently just a cell culture-derived phenomenon that has yet to be observed in a primary isolate, but it can still have an impact on surveillance and vaccine selection, which rely on the propagation of primary isolates in cell culture.

Reduced receptor binding avidity of H3N2 viruses presents difficulties in vaccine manufacturing and production

Another complication presented by the poor growth of H3N2 viruses in cell culture and eggs, is the effect it has on vaccine manufacturing and production. Although MDCK-SIAT1 cells appear to be a good alternative to MDCK cells for cell culture propagation, egg-based propagation still requires adaptive mutations for H3N2 viruses to grow. And because almost all influenza vaccines are currently produced in eggs, this limits the effectiveness of H3N2 vaccines. For example, the choice of the H3N2 vaccine component for the 2015-2016 influenza season was heavily influenced by which clade of H3N2 viruses could grow well in eggs. Although HA antigenic clade 3C.2a was dominating circulation, a virus from HA antigenic clade 3C.3a was chosen as the vaccine strain (Anonymous, 2015). This is because the HA Y159+T160 epitope present in HA antigenic site B of Clade 3C.2a viruses cannot grow in eggs, consistent with our findings that we were unable to rescue and grow the A/Texas/50/2012-F159Y+K160T mutant virus in Chapter 3 without it acquiring mutations. When grown in eggs, these viruses

quickly acquire mutations at HA residue 160, resulting in a loss of a glycosylation at residue 158, thus drastically altering the antigenicity of HA antigenic site B in these viruses (Chambers et al., 2015). Egg-adaptive mutations that emerge during vaccine production are a common concern and have even been implicated as the reason for the H3N2 vaccine mismatch during the 2012-2013 influenza season (Skowronski et al., 2014). One way around this issue is to focus more on cell-based vaccine production. Recent work shows that MDCK-SIAT1 cells can be successfully used to produce high quality vaccines without mutations (Abdoli et al., 2016; Oh et al., 2008). Although this would be a difficult shift for vaccine producers, it would likely result in a more reliable, uniform, and effective influenza vaccine.

The immunodominance of HA antigenic site B in neutralizing Ab responses against H3N2 influenza viruses and the possible role of glycosylation

Influenza viruses constantly accumulate mutations in Ab binding epitopes scattered around the globular head of HA in order to escape prior immunity and continue circulating. This process is termed antigenic drift and is responsible for the vaccine strain updates that are required every few years for each influenza subtype. In Chapter 3, we identified a mutation that emerged in H3N2 viruses during the 2014-2015 influenza season and resulted in a vaccine mismatch. The mutation was located in HA antigenic site B, and accounted for almost all of the antigenic distinction between the 2014-2015 vaccine strain and the newly emerged 2014-2015 viruses. HA antigenic site B has been repeatedly implicated as an important site for antigenic drift mutations throughout the evolution of H3N2 viruses (Koel et al., 2013; Popova et al., 2012). Although most of the

previous work has been done utilizing antisera collected from previously naïve ferrets infected for the first time with influenza, our study demonstrated that the majority of human Ab responses against current H3N2 viruses are also directed against HA antigenic site B.

So why is HA antigenic site B such an immunodominant epitope for Ab responses against H3N2 viruses? The term immunodominant refers to the majority of the response being focused on a specific epitope (Popova et al., 2012; Rajnavolgyi et al., 1997). One hypothesis regarding the importance of HA antigenic site B is that this antigenic site is one of the few sites on H3 viruses that are not glycosylated (Meyer and Wilke, 2015). H3N2 viruses have continuously gained glycosylation sites in HA since being introduced into humans in 1968 (Suzuki, 2011; Tate et al., 2011; Vigerust et al., 2007). The large N-linked glycosylations attached to the HA surface present a high level of steric hindrance to binding Abs (Pentiah et al., 2015; Tate et al., 2014). Only the HA surfaces left open and exposed can act as Ab binding epitopes, thus focusing the Ab response to specific epitopes over time as more glycosylation sites are added. This fits well with the hypothesis we made in Chapter 4 regarding the sharp decline of K166 HA-specific H1N1 Ab responses in humans born after 1986. A new glycosylation site was added to sH1N1 viruses beginning in 1986 and it is predicted to act as an umbrella-like shield that covers and hides the K166 HA-epitope from Ab binding. Multiple studies have already implicated the addition of glycosylation sites in antigenic change and abrogation of Abs that previously bound to HA (Tate et al., 2014). For example, inserting two or four additional glycosylation sites into the 1968 H3N2 pandemic strain reduced neutralizing Ab titers and increased immunopathology compared to the WT virus in previously naïve

mice (Wanzeck et al., 2011). Therefore, it is likely that glycan shielding plays an important role in limiting the antigenic sites available to anti-H3N2 Abs.

Other possible explanations of the HA antigenic site B immunodominant response against H3N2 viruses include the location of site B directly at the top of the HA head and its proximity to the receptor binding site. It is clear that future influenza virus surveillance should monitor H3N2 viruses for the emergence of new mutations in HA antigenic site B that could signal antigenic drift. This approach of closely monitoring HA antigenic site B mutations can even be taken a step further. Multiple groups have recently shown that growth of a historical influenza virus *in vitro*, in the presence of polyclonal sera, will actually elicit escape mutations that recapitulate the antigenic drift that has occurred in nature (DeDiego et al., 2016; Li et al., 2016). Passaging of the H3N2 virus A/Victoria/361/2011 in the presence of polyclonal human antisera elicited mutations in HA antigenic site B at residue 156, the exact residue that was mutated in antigenically drifted H3N2 viruses of the 2012-2013 influenza season (DeDiego et al., 2016). Li and colleagues also concluded that residues 153-156 in HA antigenic site B play a key role in influenza antigenicity (Li et al., 2016). By growing the current H3N2 vaccine strain in the presence of polyclonal human sera, we may be able to predict future mutations that will arise in HA antigenic site B. This information could then be used to create antigenically drifted vaccine strains. This work in association with recent observations of "back-boosting," where infection with an antigenically drifted H3N2 virus actually boosts Ab titers even higher against previously seen strains (Fonville et al., 2014), may present a new vaccination strategy: using escape mutations to generate artificially drifted influenza strains, and then using a prime-boost vaccine regimen where the currently circulating

strain is the first vaccination and the artificially drifted strain is the second vaccination. This would in theory boost titers against the currently circulating strain even higher than multiple vaccinations with just the current strain.

However, it is important to remember that antigenic sites other than HA antigenic site B may be important for H3N2 antigenic drift as well. Similar to Ab responses elicited against pH1N1 viruses, anti-H3N2 Ab responses may have different specificities in different individuals due to unique pre-exposure histories. This topic will be discussed in more detail later in this chapter.

The human Ab response against pH1N1 viruses is determined by pre-exposure history to sH1N1 viruses

While the majority of human anti-H3N2 Ab responses are focused on a single antigenic epitope, human anti-H1N1 Ab responses can be targeted to multiple antigenic sites on the HA surface. The exact target site is determined by an individual's pre-exposure history and the conserved epitopes shared between previous strains and the currently infecting strain (Li et al., 2013b; Linderman et al., 2014). Additionally, more antigenic sites are available on the surface of H1 viruses because there are less glycosylation residues present (Zhang et al., 2013b). As discussed in the previous section, H3 viruses are heavily glycosylated (Suzuki, 2011; Tate et al., 2011; Vigerust et al., 2007) and HA antigenic site B may be the only epitope accessible for Ab binding (Meyer and Wilke, 2015; Pentiah et al., 2015; Tate et al., 2014). Recent H1 viruses, on the other hand, have very few glycosylation sites. In fact, the pH1N1 viruses that emerged in 2009 contained only a single potential glycosylation site on the HA head (Job et al., 2013).

Studies have demonstrated that the addition of more glycosylation sites into the pH1N1 head results in resistance to neutralizing Abs (Job et al., 2013) and that different glycosylation sites can alter antigenicity of H1N1 viruses (Sun et al., 2013). However, there are currently very few potential glycosylation sites in circulating pH1N1 strains, resulting in a wide array of exposed Ab binding epitopes across the HA head.

The data presented in Chapter 4 demonstrates that different sH1N1 pre-exposure histories elicit unique Ab responses against distinct HA epitopes of the pH1N1 virus. Different aged individuals within the human population have unique Ab specificities (Li et al., 2013b; Linderman et al., 2014). Individuals are infected early in life by H1N1 viruses that circulate during their respective childhoods. When they are challenged later in life with an antigenically distinct pH1N1 virus, there are a limited number of conserved epitopes shared between the current pH1N1 virus and the sH1N1 virus seen early in life. Memory B cells specific for conserved epitopes are recalled and produce the majority of the Abs in the secondary response. Humans born during different decades have been exposed to different sH1N1 viruses early in life, thus explaining why different age groups elicit Ab responses with different specificities against pH1N1 viruses.

H1N1 pre-exposure history elicits a unique Ab response in middle-aged adults against pH1N1 virus that correlates with higher susceptibility to infection

Influenza surveillance and vaccine strain selection have always relied heavily on the use of antisera collected from previously naïve ferrets infected only one time with influenza virus. Previous work from our lab has demonstrated that Abs elicited in previously naïve ferrets infected with the 2009 pH1N1 virus a single time do not have the

same specificity as anti-pH1N1 Abs elicited in young adults (born 1983-1996) (Li et al., 2013b). The data presented in Chapter 4 demonstrates that the use of sera collected from previously naïve ferrets infected with a single influenza virus is also leading to the antigenic mischaracterization of newly emerged HA mutations. Specifically, we found that a K166Q HA mutation that became fixed in pH1N1 viruses during the 2013-2014 influenza season abrogates the binding of Abs in the sera of a large portion of middle-aged adults (born 1965-1979). It was hypothesized that this Ab specificity for the HA epitope including residue K166 is present in middle-aged adults because the K166 HA-epitope is conserved between the pH1N1 virus of 2009 and the sH1N1 strains that circulated during their childhoods (the 1970s and early 1980s). To test this hypothesis, we sequentially infected ferrets with a sH1N1 virus from 1983 and then the 2009 pH1N1 virus. We found that these animals mounted Abs that recognized the HA epitope involving residue K166 that is conserved between the 1983 sH1N1 virus and the 2009 pH1N1 virus. The functional consequence of these K166 HA-specific Abs was also determined using passive transfer experiments in mice where mAbs specific for the epitope involving K166 did not protect mice against viruses possessing Q166.

This conclusion that Abs specific for the K166 HA-eptitope make the individual more susceptible to severe infection correlates well with the observation that middle-aged adults experienced a drastically increased burden of severe influenza infections during the 2013-2014 season. According to WHO reports, middle-aged individuals (defined by the WHO as 18-64 years old) experienced 61.2% of influenza-related hospitalizations and 62% of influenza-related deaths during the 2013-2014 influenza season (Arriola et al., 2014). Typically, those values are between 20-35% for an individual influenza season.

The majority of severe influenza infections usually affect those with weaker immune systems: the very young (0-4 years old) and the elderly (>65 years old). We recently completed additional experiments where we defined the specificity of sera collected from 323 individuals prior to the 2013-2014 influenza. We found that individuals that possessed Abs focused on the K166 HA-epitope were more prone to pH1N1 infection during the 2013-2014 influenza season (Petrie et al., under review).

Identification of the unique anti-pH1N1 Ab responses in other age groups

Our group has now identified the specificities of anti-pH1N1 Abs that are common among individuals in two separate age groups. As explained above, the unique pre-exposure history of individuals in each age group to different sH1N1 viruses early in life led to different anti-pH1N1 Ab responses. Young adults (born 1983-1996) possess an Ab response targeted to the HA epitope involving residue K133 (Li et al., 2013b). Middle-aged adults (born 1965-1979) possess an Ab response targeted against the K166 HA-epitope (Huang et al., 2015; Linderman et al., 2014). The epitope involving K133 was present in sH1N1 viruses circulating between 1983-1996 and the epitope involving K166 was present in sH1N1 viruses circulating prior to 1983.

It is possible that there are different Ab specificities that are common among individuals in other age groups as well. For example, it would be very interesting to investigate the anti-pH1N1 Ab specificity of children. School-aged children have been implicated as the main population responsible for transmission during annual influenza circulation (Petrie et al., 2013; Viboud et al., 2004). Being the main population for transmission would exhibit a large immune pressure that may have already forced the

virus to adapt and mutate to avoid the unique Ab specificity present in young children. However, none of the mutations that have emerged in pH1N1 viruses since 2009 appear to have an effect on the Ab binding of sera isolated from influenza-exposed children (unpublished data from our lab). Likewise, although the Ab specificity of young adults is against the epitope involving HA residue K133, this epitope has not yet acquired a mutation in circulating pH1N1 viruses. There could be many forces at play that we do not yet understand on how influenza viruses continuously circulate. Different age groups may allow for transmission in different geographical locations, or each age group may be partially responsible for circulation. Nonetheless, the population that controls the majority of transmission and circulation should have the largest immune pressure on forcing antigenic drift mutations in the pH1N1 HA gene segment.

Do these unique secondary Ab responses represent original antigenic sin?

Pre-exposure history has been shown here to elicit unique Ab responses against pH1N1 influenza virus in different age groups of the human population. The specificity of these unique Ab responses is determined by the HA epitopes conserved between the sH1N1 strains these individuals were infected with early in life and the pH1N1 virus they are being challenged with now. Whenever a secondary Ab response is altered and characterized depending on previous infections, it is necessary to establish whether original antigenic sin (OAS) is playing a role. In general, OAS describes the observation that even when infected with a novel influenza strain, humans will produce Abs against previously encountered strains at the expense of mounting effective responses against the current novel strain (Davenport et al., 1953; Francis, 1960). In that sense, our

observations of different pre-exposure histories leading to unique Ab repertoires would not fall under the category of OAS for one major reason: there is no so-called "sin" observed in our studies. Take the middle-aged adult age group with Abs specific for the K166 HA-epitope for example. Although these individuals preferentially mount an Ab response that is specific for an epitope present in the sH1N1 strains they saw early in life, they still mount sufficiently high responses against the A/California/07/2009 pH1N1 strain. If this were an example of OAS, the Ab response would be high against past sH1N1 strains at the expense of high titers against the currently infecting strain. As long as the K166 HA-epitope is intact, the Ab response against the current strain is protective. It's only when HA residue 166 is mutated that this K166 HA-specific Ab response is deleterious. It remains to be seen whether viruses possessing Q166 recall Ab responses that target the K166 HA-epitope in individuals with sH1N1 pre-exposures from the early 1980s.

Another question along the lines of OAS to consider is whether an individual's first influenza exposure is really the most important in shaping lifelong immunity. An alternate way to look at this is whether these unique Ab responses we have discovered in different age groups are actually fully dependent on the epitopes conserved between the current pH1N1 strain and the very first sH1N1 strain with which they were infected. So not just "strains they saw early in life," but rather "their very first sH1N1 infection." This could help explain why only 42% of our middle-aged adult sera possessed Abs sensitive to the K166Q mutation. The individuals without this Ab specificity for the K166 HA-epitope may have been first infected with an A/USSR/90/1977-like virus which does not have the K166 HA-epitope intact. Even if they were later infected with a sH1N1 strain

with an intact K166 HA-epitope (like A/Chile/1/1983), they would still not mount an Ab response specific for the K166 HA-epitope. Only those infected for the first time with a sH1N1 virus with the K166 HA-epitope intact would produce this unique specificity later in life. Previous studies have suggested that an individual's first influenza exposure confers an antigenic seniority to their response, which is continually boosted with repeated sequential infections (Kucharski et al., 2015; Lessler et al., 2012; Miller et al., 2013). Overall, the unique Ab responses mounted against pH1N1 viruses in different age groups generally do not represent true OAS. Although the specificity of the response is dictated by sH1N1 strains seen early in life, the response is still sufficient enough to protect against the current pH1N1 strain. Only when a mutation arises in that specific epitope does sin truly occur in that the response is highly effective against past strains, but low in effectiveness against the current strain.

Does pre-exposure history have as much of an impact on shaping the anti-H3N2 influenza response?

Our data suggests that pre-exposure to different sH1N1 strains can affect the Ab specificity against pH1N1 infections later in life. This presents a new factor that must be taken into account during vaccine selection and antigenic characterization of newly emerged mutations. However, it will also be important to determine whether pre-exposure history shapes unique Ab responses against subsequent H3N2 influenza infections as well. One major difference between H1N1 and H3N2 viruses is the level of antigenic distinction between the strains seen early in life and those currently circulating. Although sH1N1 and pH1N1 viruses are classified as the same subtype of influenza, they

are thought of as two entirely different pandemics because pH1N1 viruses emerged after obtaining a genetically divergent HA gene segment from a classical swine lineage (Garten et al., 2009). Approximately 20-30% of the amino acid residues in HA are different between sH1N1 viruses and pH1N1 viruses (Li et al., 2012). This substantial difference, especially in the variant globular head domain, does not leave many conserved epitopes shared between sH1N1 and pH1N1 viruses. A limited availability of conserved epitopes may be required for the focusing of an immunodominant response on a specific HA epitope, similar to what we found in the middle-aged adult sera regarding the K166 HA-epitope. On the other hand, H3N2 viruses of the 1960s and 1970s are at most 8-12% different in their HA sequences compared to H3N2 viruses of today (Bedford et al., 2010; Broberg et al., 2015; Smith et al., 2004). This results in many more conserved epitopes between H3N2 strains, which may limit the ability of the secondary Ab response to be focused on a single shared epitope.

H3N2 viruses have been circulating constantly in humans since 1968. In that time they have acquired multiple mutations in each of the five antigenic sites of H3N2 HA (Smith et al., 2004). Derek Smith's group has completed elegant work to take an in-depth look at the evolution of H3N2 viruses and the requisition of antigenic drift mutations. Through the use of previously naïve ferret antisera, they identified a set of several residues in HA antigenic site B that are responsible for the majority of antigenic drift in H3N2 viruses over the past 50 years (Koel et al., 2013). However, many other mutations have become fixed in the other H3 antigenic sites as well during that time. Our hypothesis is that mutations are elicited in these other sites in order to escape other Ab responses mounted in individuals with unique H3N2 pre-exposure histories. So similar to

the H1N1 pre-exposure history observations, individuals with different H3N2 pre-exposure histories might possess anti-H3N2 Abs against different epitopes of HA; and H3N2 viruses might acquire mutations in all these epitopes in order to escape population immunity and continue circulating. So although Derek Smith and his colleagues have carried out great investigations into H3N2 antigenic drift, their answers are potentially skewed and limited by their use of only sera collected from previously naïve ferrets to antigenically characterize historic H3N2 viral isolates. Pre-exposure history in the human population could have shaped unique Ab responses to epitopes other than HA antigenic site B. This hypothesis is also supported by our human sera data from Chapter 3, where although the majority of our human sera cohort possessed Abs against HA antigenic site B, about 12.5% had high titer Ab responses targeted elsewhere on HA.

We are currently conducting studies to determine the importance of H3N2 pre-exposure history on the Ab response against gradually drifted H3N2 viruses, and how it compares to what we observed with H1N1 pre-exposure histories. As we have shown previously with H1N1 infections, ferrets sequentially infected with antigenically distinct influenza strains will recapitulate the unique Ab responses observed in humans (Li et al., 2013b; Linderman et al., 2014). For our H3N2 study, ferrets were sequentially infected with antigenically distinct H3N2 viruses in order to determine if Ab specificities would be shifted to epitopes conserved between the two strains. Preliminary data indicates a possible shift in the Ab binding epitope between different ferret groups, but more data and experiments are needed before making final conclusions regarding H3N2 pre-exposure history and the impact it has on shaping Ab responses.

Concluding Remarks

Overall, my work has investigated multiple factors that complicate viral surveillance and selection of effective vaccine strains for the annual influenza vaccine. Poorly growing H3N2 isolates acquire mutations quickly during cell culture propagation and hinder antigenic characterization, vaccine selection, and even vaccine production. Unique Ab repertoires elicited by different pre-exposure histories are not represented through the use of sera collected from previously naïve ferrets infected with influenza for the first time in current surveillance techniques. This leads to mischaracterization of the antigenic importance of newly emerging HA mutations. And as we demonstrated with the single HA antigenic site B mutation in H3N2 viruses during the 2014-2015 influenza season, even single mutations can lead to antigenic drift and complete vaccine mismatches.

Even though the future of influenza vaccination may be destined to include more universal approaches, such as recombinant proteins, peptide antigens, and DNA-based vaccines, it is still important to continue improving our current vaccination techniques. Steps are already being taken to improve viral surveillance. The adoption of a "sequence first" strategy will ensure that sequencing data matches that of circulating strains, without the danger of cell culture-adaptive mutations. The utilization of MDCK-SIAT1 cells for propagation will decrease the number of cell culture-adaptive mutations and allow recent H3N2 viruses to grow well enough for true antigenic characterization and vaccine selection. However, more steps need to be taken. The current egg-based vaccine manufacturing process results in egg-adaptive mutations during production, possible vaccine mismatches, and a limit on which viruses can be selected for inclusion in the

influenza vaccine. Adoption of a cell culture-based vaccine manufacturing process could reduce the level of variation in vaccine stocks and also allow for some viral clades with low fitness to be included when relevant.

Another vital factor in improving the influenza vaccine will be making sure that antigenic characterizations of newly emerged mutations are correct. As we have demonstrated multiple times, sera collected from previously naïve ferrets infected with influenza a single time do not detect all the antigenically important mutations that emerge. Different human age groups have different Ab repertoires against the same virus due to unique pre-exposure histories. This does not call for personalized vaccine strains based on age; that would be too difficult and too expensive to be feasible. However, what is required is a more stringent detection system that will accurately characterize the antigenic importance of every mutation. One approach that could improve antigenic characterization of newly emerged mutations would be the use of sequentially infected ferret antisera. As we have demonstrated multiple times (Li et al., 2013b; Linderman et al., 2014), ferrets infected sequentially with the same strains as humans are able to recapitulate the immunodominant specificities observed in human Ab responses. Using a set of differentially pre-exposed ferret groups, where each group is primed with a different historic influenza virus and then re-challenged with the same current strain, would hopefully allow us to represent all the unique Ab repertoires present in the human population.

An alternative approach would be to simply use human antisera raised against influenza virus. Collecting antisera from different aged humans and testing samples individually could determine the antigenic effect of a newly emerged mutation on the

entire spectrum of unique human Ab responses against the pH1N1 virus. In fact, based upon the work and the conclusions presented above, the WHO has recently (within the last month) changed the way they conduct viral surveillance and will now use human antisera to determine antigenic change moving forward (Anonymous, 2016b). Additionally, based upon the results they obtained using human antisera instead of antisera from previously naïve ferrets, the WHO has elected to update the pH1N1 vaccine strain for the first time since 2009 (Anonymous, 2016b). Using human antisera for surveillance and vaccine strain selection moving forward will help ensure that the vaccine strains are updated to more effective strains that better protect humans of all ages.

Annual vaccination against influenza virus is an important tool in fighting off a disease that causes millions of infections and thousands of deaths in the United States each year (Kostova et al., 2013; Reed et al., 2015). It is vital that the best strains are chosen each year in order to get maximum vaccine effectiveness. Sometimes, vaccine mismatches cannot be avoided due to the quick emergence of antigenically drifted viruses. However, the continued use of sera collected from previously naïve ferrets following a single influenza infection and MDCK-based cell culture propagation has been putting the field at a disadvantage by not correctly characterizing the antigenic importance of new HA mutations. Implementation of a "sequence-first" surveillance program, an MDCK-SIAT1 cell culture system for primary isolates and vaccine production, and the use of clinical human antisera for antigenic characterization of new mutations are the next steps towards improving the annual influenza vaccine.

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