Hemagglutination Inhibition Antibody Landscapes after Vaccination with diverse H7 hemagglutinin proteins

Hyesun Jang[1] and Ted Ross[1]

[1]University of Georgia

July 28, 2020

Abstract

Background: A systemic evaluation of the antigenic breadth of the H7 influenza hemagglutinin (HA) proteins, especially for the viruses isolated after 2016, are limited. The purpose of this study was to investigate the antigenic breadth of major H7 strains with an ultimate aim to discover H7 HA proteins that can elicit protective receptor-blocking antibodies against co-circulating H7 influenza strains. Method: A panel of nine H7 influenza strains were selected from 3,633 H7 HA amino acid sequences identified over the past two decades (2000-2018). The sequences were expressed on the surface of virus like particles (VLPs) and used to vaccinate C57BL/6 mice. Serum samples were collected and tested for hemagglutination-inhibition (HAI) activity. The vaccinated mice were challenged with lethal dose of H7N9 virus, A/Anhui/1/2013. Results: VLPs expressing the H7 HA antigens elicited broadly reactive antibodies each of the other vaccine strains, except the A/Turkey/Italy/589/2000 (Italy/00) H7 HA. The putative N-glycosylation site at antigenic site B was identified as a unique antigenic profile of Italy/00. Introduction of the putative glycosylation site (H7 HA-A169T) significantly altered the antigenic profile of HA from the A/Anhui/1/2013 (H7N9) strain. Conclusion: This study focused the importance of key amino acid mutations that result in severe vaccine mismatches for future H7 epidemics. Future universal influenza vaccine candidates will need to focus on viral variants with these key mutations.

Hemagglutination Inhibition Antibody Landscapes after Vaccination with diverse H7 hemagglutinin proteins

Hyesun Jang [1], and Ted M Ross[1,2]

[1]Center for Vaccines and Immunology,[2]Department of Infectious Diseases

University of Georgia, Athens, GA, USA

[*]To whom correspondence shall be addressed:

Ted M. Ross, Ph.D.

Center for Vaccines and Immunology

University of Georgia

CVI Room 1504

501 D.W. Brooks Drive

Athens, GA 30602

Tel: 706-542-9708

Fax: 706-583-0297

Email: tedross@uga.edu

#To whom correspondence shall be addressed:

Abstract: 213 words

Main text: 4192 words

Running Title: Antibody cross-reactivity of H7Nx influenza HA proteins

Keywords: antibodies, vaccines, antigenicity, hemagglutinin, H7

Abstract (maximum 250 words)

Background: A systemic evaluation of the antigenic breadth of the H7 influenza hemagglutinin (HA) proteins, especially for the viruses isolated after 2016, are limited. The purpose of this study was to investigate the antigenic breadth of major H7 strains with an ultimate aim to discover H7 HA proteins that can elicit protective receptor-blocking antibodies against co-circulating H7 influenza strains.

Method: A panel of nine H7 influenza strains were selected from 3,633 H7 HA amino acid sequences identified over the past two decades (2000-2018). The sequences were expressed on the surface of virus like particles (VLPs) and used to vaccinate C57BL/6 mice. Serum samples were collected and tested for hemagglutination-inhibition (HAI) activity. The vaccinated mice were challenged with lethal dose of H7N9 virus, A/Anhui/1/2013.

Results: VLPs expressing the H7 HA antigens elicited broadly reactive antibodies each of the other vaccine strains, except the A/Turkey/Italy/589/2000 (Italy/00) H7 HA. The putative$N$ -glycosylation site at antigenic site B was identified as a unique antigenic profile of Italy/00. Introduction of the putative glycosylation site (H7 HA-A169T) significantly altered the antigenic profile of HA from the A/Anhui/1/2013 (H7N9) strain.

Conclusion: This study focused the importance of key amino acid mutations that result in severe vaccine mismatches for future H7 epidemics. Future universal influenza vaccine candidates will need to focus on viral variants with these key mutations.

Introduction

Avian-origin influenza A hemagglutinin subtype 7 viruses (H7 AI viruses) circulate primarily in avian hosts. Humans are dead-end hosts for these virus infections and the H7 epidemics rarely persist among humans. However, some H7 influenza viruses may mutate in the human respiratory track and cause severe recurring epidemics[1]. There have been five epidemics caused by Asian H7N9 influenza viruses between 2013-2018 and this raises concern that this subtype may have the potential to cause recurring influenza virus pandemics[2-4]. H7N2 influenza viruses caused epidemics in 2002 and 2003 and silently circulated among feline species and/or unknown reservoirs for fourteen years [5]. In the northeastern U.S., H7N2 influenza viruses have high affinity for the mammalian respiratory tract and are highly adapted to mammalian species with increased affinity toward α2-6 linked sialic acid [6]. In 2016, the feline H7N2 influenza viruses infected attending veterinarians from shelter cats by reverse-zoonotic infection[7]. Even without adaptation, H7 influenza virus strains have caused at least four human epidemics since 2000: 1) the H7N1 influenza viruses infected people in Italy, 2) the H7N2 influenza viruses infected people in Northeastern U.S., 3) two distinct H7N3 influenza viruses infected people in North American and Eurasian countries, 4) and people in Europe were infected with H7N7 influenza viruses. These epidemics demonstrate the potential that another avian influenza virus of the H7 subtype may infect and begin transmitting between humans to initiate the next H7 influenza virus pandemic.

For prompt production and distribution of vaccines during a pandemic emergency, the World Health Organization (WHO) has stockpiled candidate vaccine viruses (CVVs) for all H7 influenza viruses[8]. However, the antigenic breadth of stockpiled CVVs have not been investigated, especially for the relatively recent H7N9 virus isolated in 2016-2017 [9]. To prepare for the next H7 influenza virus epidemics, it is imperative to identify the antigenic breadth of co-circulating H7 HA proteins and clarify the target coverage by the antigen.

There have been a small number of studies that investigated the antigenic breadth of multiple H7 strains. Vaccination with divergent H7 HA immunogens isolated in 2009 from North American or Eurasian H7Nx viruses elicit immune responses that protect against Asian H7N9 influenza viruses[10]. Anti-H7 HA antiserum recovered from humans vaccinated with Anhui/13 H7 HA recombinant protein has broad binding activity to diverse H7 strains, including A/feline/New York/16-040082-1/2016 (H7N2) and to H7 HA from the A/turkey/Indiana/16-001403-1/2016 (H7N8) virus [11]. There were strong two-way cross-reactivity among H7N9, H7N2, H7N3 and H7N7 influenza viruses [12]. However, it is difficult to draw conclusions about the overall antigenic breadth of co-circulating H7 influenza strains since each study used different representative reference strains expressed from different formats, In addition, these H7 HA antigens were isolated prior to 2016 and did not represent the current H7 HA variants. In this study, we primarily investigated the antigenic breadth of H7 influenza HA proteins that co-circulated in human over the last two decades.

Materials and Methods

Clustering of HA amino acids sequences and vaccine preparation

The H7 HA amino acid sequences uploaded on Global Initiative on Sharing All Influenza virus Data (GISAID) from 2000 to 2018 were downloaded. The sequences were aligned using Geneious software (Auckland, New Zealand). The amino acids 20-300 (HA1) region were extracted and partial or duplicate sequences were eliminated. The sequences were divided into three time periods/searches (2000-2012, 2013-2018 and 2013-2018 non-H7N9 sequences). The trimmed HA1 sequences of each group was subjected to MUSCLE alignment and clustered by 97% identity. Each cluster was illustrated as a pie chart using PRISM GraphPad Software (San Diego, CA, USA) and a panel of nine H7 strains of each cluster was selected.

Total of nine H7 HA sequences were expressed on the surface of virus like particle (VLPs), as previously described [13]. Briefly, the full-length H7 HA amino acid sequences were subjected to codon optimization for expression in human (Genewiz, Washington, DC, USA) and inserted into the pTR600 expression vector. The plasmid encoding H7 HA were transiently co-transfected with plasmids expressing HIV-1 Gag (optimized for expression in mammalian cells), NA (A/Thailand/1(KAN-1)/2004 H5N1) (optimized for expression in mammalian cells). The cells were incubated for 72 h at 37°C (Medigen Inc., Rockville, MD, USA). Supernatant was centrifuged in low speed and filtrated through a 0.22-μm sterile filter. Filtered supernatant was purified via ultracentrifugation (100,000 g through 20% glycerol, weight per volume) for 4h at 4°C. The pellets were subsequently resuspended in PBS (pH 7.2) and stored in single-use aliquots at 4°C until use.

The HA content of H7 VLPs was determined as previously described with slight modification [14]. Briefly, A high-affinity, 96-well flat bottom enzyme-linked immunosorbent assay (ELISA) plate was coated with 5 to 10 g of total protein of VLPs and serial dilutions of a recombinant H7 antigen (A/Anhui/1/213 HA generated in house) in ELISA carbonate buffer (50 mM carbonate buffer, pH 9.5), and the plate was incubated overnight at 4°C. The next morning, plates were washed in PBS with 0.05% Tween 20 (PBST), and then nonspecific epitopes were blocked with 1% bovine serum albumin (BSA) in PBST solution for 1 h at room temperature (RT). Buffer was removed, and then stalk-specific group 2 antibody (CR8020) was added to the plate and incubated for two hours at 37°C. Plates were washed and probed with goat anti-human IgG horseradish peroxidase-conjugated secondary antibody at a 1:3000 dilution and incubated for 2 h at 37°C. Plates were washed 7 times with the wash buffer prior to development with 100 μL of 0.1% 2,2'-azinobis(3-ethylbenzothiaozoline-6 –sulphonic acid; ABTS) solution with 0.05% H_2O_2 for 40 min at 37°C. The

reaction was terminated with 1% (w/v) sodium dodecyl sulfate (SDS). Colorimetric absorbance at 414 nm was measured using a PowerWaveXS (Biotek, Winooski, VT, USA) plate reader. Background was subtracted from negative wells. Linear regression standard curve analysis was performed using the known concentrations of recombinant standard antigen to estimate the HA content in VLP lots.

Mouse study

C57BL/6 mice (*Mus musculus* , females, 6 to 8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in microisolator units. The mice were allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Mice (8 mice per group) were intramuscularly injected 2X at four-week intervals with each vaccine (HA content=3 μg) with AddaVax^{TM} adjuvant (Invivogen, San Diego, CA, USA) (Figure 2). Mice were bled at week 8 and transferred to a biosafety level 3 (BSL-3) facility for viral challenge. At week 12, mice were briefly anesthetized and infected with a 100 LD_{50} dose of A/Anhui/1/2013 H7N9 via intranasal route ($1X10^3$PFU/0.05 ml) (Figure 2). At 4 days post-challenge, three mice in each group were randomly selected and sacrificed to harvest lung tissue (Figure 2). Remaining mice were monitored for clinical symptoms and euthanized at 12 days post-challenge (Figure 2). All procedures were in accordance with the NRC Guide for Care and Use of Laboratory Animals, the Animal Welfare act, and the CDC/NIH Biosafety and Microbiological and Biomedical Laboratories (IACUC # A2017 11-021-Y3-A11).

Hemagglutination-Inhibition (HAI) assay

To evaluate the humoral response to each vaccination, blood was collected via submandibular bleeding using a lancet and transferred to a microfuge tube. Tubes were incubated at room temperature for at least 30 min prior to centrifugation, sera were collected and frozen at -20 °C +- 5 degC. A hemagglutination inhibition assay (HAI) assay was used to assess receptor-blocking antibodies to the HA protein to inhibit agglutination of turkey red blood cells (TRBCs). The protocol is taken from the CDC laboratory influenza surveillance manual. To inactivate non-specific inhibitors, mouse sera was treated with receptor destroying enzyme (RDE, Denka Seiken, Co., Japan) prior to being tested. Three parts of RDE was added to one-part sera and incubated overnight at 37degC. The RDE was inactivated at 56degC for 30 min; when cooled, 6 parts of sterile PBS was added to the sera and was kept at 4 degC until use. RDE treated sera was two-fold serially diluted in v-bottom microtiter plates. Twenty-five μl of VLPs at 8 HAU/50 μL was added to each well (4 HAU/25 μL). Plates were covered and incubated with virus for 20 min at room temperature before adding 0.8% TRBCs in PBS. The plates were mixed by agitation and covered; the RBCs were then allowed to settle for 1 h at room temperature. HAI titer was determined by the reciprocal dilution of the last well which contained non-agglutinated RBC. Negative and positive serum controls were included for each plate. All mice were negative (HAI < 1:10) for pre-existing antibodies to currently circulating human influenza viruses prior to study onset.

Plaque forming assay (PFA)

Viral titers were determined using a plaque forming assay as previously using 1×10^6 Madin-Darby Canine Kidney (MDCK) cells, as previously described [15]. Briefly, lung samples collected at 4 days post challenge were snapped frozen and kept at -80 degC until processing. Lungs were diluted ($10^0$to 10^5) and overlayed onto confluent MDCK cell layers for 1 h in 200 μl of DMEM supplemented with penicillin–streptomycin. Cells were washed after 1-hour incubation and DMEM was replaced with 3 mL of 1.2% Avicel (FMC BioPolymer; Philadelphia, PA) - MEM media supplemented with 1μg/mL TPCK-treated trypsin. After 48 h incubation at 37 °C with 5% CO2, the overlay was removed and washed 2x with sterile PBS, cells were fixed with 10% buffered formalin and stained for 15 mins with 1% crystal Violet. Cells were washed with tap water and allowed to dry. Plaques were counted and the plaque forming units calculated (PFU/mL).

Determination of HAI cut-off to predict protection against challenge.

The Receiver-operation curve (ROC) analysis between HAI titer and protection against Anhui/13 challenge,

as previously described[16]. The protection was defined when the mouse could maintain 95% the minimum during entire challenge study. Based on four cut-off values (VLP HAI titer=40, 80,160, and 320), a positive or negative prediction was determined by comparing the protection to the HAI titer. The sensitivity was calculated as "#of mouse which showed hemagglutination inhibition (HAI) titer [?] cut-off and was protected from the challenge study/ # of all protected mice". The Specificity was calculated as #of mouse which showed hemagglutination inhibition (HAI) titer < cut-off and unprotected from the challenge study/ # of all unprotected mice. The ROC curve was generated by connecting plots of sensitivity% versus 100-specificity% (false positive). The area under the curve (AUC) and Youden's index (Sensitivity + Specificity -1) was calculated by Prism (Graphpad software). The optimal cut-off was determined based on highest AUC or Youden's index to be used as a surrogate of protection.

Site directed mutagenesis.

The H7 HA numbering was based on previous report [17]. The amino acids at residues 167 to 170 were changed from NAAF to NATF in the putative antigenic site B of A/Anhui/1/2013 H7N9 HA. The NATF amino acids are located at this position in the A/Turkey/Italy/589/2000 H7N1 HA molecules. By the single amino acid substitution, it is expected to introduce N-glycosylation site to the antigenic site B, located nearby the receptor binding site. The plasmid was expressed as VLPs as described above.

Statistical analysis.

The difference in serum HAI titer and lung viral titer among groups was analyzed by ordinary one-way ANOVA, followed by Tukey's multiple comparison test. The difference in body weight loss of each time point was tested by Repeated Measures one-way ANOVA followed by Tukey's multiple comparison test. All statistical analysis was performed using Prism GraphPad Software.

Results

Phylogenetic analysis of H7Nx viruses isolated in between 2000 and 2018

Among the 3,363 amino acid sequences uploaded to GISAID, almost half of the sequences (1740) showed 97% or higher HA1 amino acid similarity to A/Anhui/1/2013 H7N9 virus (Anhui/13-like). The uploaded amino acid sequences were biased to isolates from Asian H7N9 epidemics, which resulted in human infection cases during epidemics between 2013-2017. Since the Anhui/13-like sequences skewed the overall phylogenetic analysis, the sequences were separately aligned in three ways: 1) sequences isolated between 2000-2012 before the emergence of Asian H7N9 (Fig. 1A), 2) H7N9 sequences isolated from 2013-2018 (Fig. 1B), and non-H7N9 sequences isolated from 2013-2018 (Fig. 1C).

Prior to the Asian H7N9 influenza virus outbreaks, the Eurasian and North American lineages represented the majority of H7 HA sequences in the database (53.14% and 45.95%, respectively) (Fig. 1A). Interestingly, most of the Eurasian H7Nx influenza viruses isolated between 2000 to 2018, had high HA amino acid similarity (95% or more) to the oldest strain in our panel, A/Mallard/Netherland/12/2000 H7N3 (Table 1). Instead of a slow drift of HA1 amino acid sequences, genetic diversification of the H7Nx influenza viruses was driven by genetic reassortment that resulted in each cluster sharing unique neuraminidase subtypes (N1, N3, N7, N9). The North American lineage influenza viruses isolated between 2000-2012 were further subdivided into two distinct clusters that shared 92.5% amino acid similarity to each other (green and yellow segments in Fig. 1A). During this 12-year period, the North American H7N3 influenza viruses had less genetic drift (<3%) and did not evolve into divergent subtypes. The North American H7N2 influenza viruses spiked only in epidemics in early 2000s (2000-2003) and were not detected thereafter.

The majority of viral sequences isolated from 2013-2018 were Anhui/13-like H7N9 influenza viruses (Fig. 1B). Approximately 5% of the HA1 sequences had 3-5% difference in the amino acid sequence and represented as a separate clusters from Anhui/13-like HA sequences (Fig. 1B). This small cluster of HA sequences consisted

of the A/Guangdong/17SF003/2016 H7N9 (Guangdong/16)-like viruses, which evolved from Anhui/13 and clustered into a separate lineage in 2016-2017. Another separate phylogenetic cluster of Asian H7N9 viruses was the A/Shanghai/1/13 H7N9 (Shanghai/13)-like viruses. The Shanghai/13 was one of the earliest human H7N9 isolates in May 2013, which evolved into a separate phylogenetic cluster from Anhui/13-like viruses[18,19]. In this sequence analysis, the Shanghai/13 virus itself belonged to Anhui/13-like virus due to high homology (98.39%) of the HA amino acid sequences. However, the derivatives of Shanghai/13 had divergent sequences to form a separate cluster that occupies ˜1% of the overall HA sequences (Fig. 1B).

The majority of non-Asian H7N9 influenza strain sequences uploaded on GSAID database between 2013 and 2018 were North-American H7N3 influenza virus derivatives, which represented ˜26% of the HA amino acid sequences prior to the 2013 Asian H7N9 influenza virus outbreaks (Fig. 1C). Most of the North American H7 influenza viruses were H7N3 viruses designated into four distinct HA sequence clusters. The A/American green-winged teal/CA/2015 H7N3 virus, which is the representative strain of the second largest cluster, is most likely derived from the H7N3 A/Bluewingteal/Ohio/658/2004 (Ohio/04) isolate. Interestingly, the northeastern U.S H7N2 strains have been rarely detected since 2004, except for one incident at an animal shelter in 2016 [7]. There are only 10 isolates that belong to the Eurasian lineage, but this is most likely due to the sampling bias for Asian H7N9 isolated in most Asian countries during that time period. All ten isolates had high homology to the NL/00 (H7N3) influenza virus.

Selection of H7 panel strains

The panel of H7 influenza strains were selected to represent the antigenic diversity of H7Nx viruses during the last two decades. Asian H7N9 strains that are known to be antigenically distinct from each other were selected [8]. For non-Asian H7N9 strains, three Eurasian strains and two North American strains were selected based upon remoteness in geography and time of isolation (Table 1). The amino acid homology ranged between 1.61-5.14%, among Eurasian strains despite of dispersed isolation and time points of collection. The North American strains shared ˜81-86% amino acid homology with Eurasian strains. Even though the Ohio/04 and New York/03 strains were isolated within a year from geographically similar regions, they shared 92.5% of the same HA amino acids. The putative antigenic site was conserved across all nine strains in the panel (Table 2). Of note, the hallmark mutation that causes N-linked glycosylation in antigenic site B was observed from Italy/00 (Table 2, blue-color coded and asteroid).

Cross-reactivity of H7 panel strains to Anhui/13 H7N9 virus

In order to determine the elicited antibody cross-reactivity between H7 strains, mice were vaccinated with virus-like particles expressing the wild-type H7 HA protein from A/Anhui/1/2013 on the surface of the particle (Fig. 2). All vaccinated mice had high titer antibodies with HAI activity against the 7 of the 8 H7N9 influenza viruses in the panel except against the NY/02 virus (Fig. 3A). The HAI titer against live H7 viruses showed similar pattern, albiet with lower titers (Fig 3B). All mice had HAI activity $>$ 1:40 against the Anhui/13 and Hunan/16 viruses. The level of cross-reactivity did not directly correlate with the antigenic similarity (Table 1 and Fig. 3).

Following challenge with Anhui/13, mice were observed for sign of morbidity and mortality (Fig. 4). Mock vaccinated mice lost greater than 15% body weight by day 7 post-infection, which was similar to mice vaccinated with NY/02 VLPs (Fig. 4A) with 60% of the mice reaching clinical endpoints and were sacrificed (Fig. 4B). Mice vaccinated with Jiangxi/09 or Gg/16 lost 12% body weight. Mice vaccinated with the other VLPs lost between 5-8% body weight, except for mice vaccinated with Hun/16 that maintained their average body for the entire challenge period. Most mice survived challenge (Fig. 4B). One most died in the Jiangxi/09 group and 2 mice died in the Gg/16 group. The lung viral titer inversely correlated to AA similarity to Anhui/13, except SH/13 (Fig. 4C and Table 1). Little to no virus was detectable in the lungs

of mice vaccinated with Anhui/13 or Shanghai/13, and only one mouse in the Hun/16 group had detectable virus (Fig. 4C).

Cross-reactiveness amongst all H7 panel strains

Since it is not feasible to conduct challenge studies against all H7 panel strains, VLP HAI titer was applied to estimate each vaccine's protective efficacy. First, ROC curve analysis was conducted between HAI titer and weight loss data following Anhui/13 challenge study (Suppl. Fig. 1). The selection of the cut-off was determined by two criteria: maximizing sensitivity (AUC of the curve) and maximized the summation of sensitivity and specificity (Youden's indect) [20]. The highest sensitivity of the prediction was observed as the maximum area under the curve when the VLP HAI cut-off was 1:80 (Suppl. Fig. 1B). The Youden's index (specificity + sensitivity -1) was highest when the HAI cut-off was 1:160 (Suppl. Fig. 1C). Thus, we used the range of 1:80-1:160 as the cut-off of HAI titer that can provide protection against a stringent challenge by each H7 influenza virus in panel. When applying the cut-offs determined by the ROC analyses, the pre-challenge HAI titer appears to correctly predict the level of protection in weight loss (Fig. 4A and Fig 5A) in a stringent Anhui/13 challenge.

Antisera elicited by each H7N9 VLPs in the panel had a broad-range of cross-reactive antibodies (Fig 5). The cross-reactivity of each antisera did not correlate with the amino acid sequence similarity of the HA (Table 1 and Fig. 5). Mice vaccinated with the four Asian H7N9 strains (Anhui/13, Shanghai/13, Guangdong/16, and Hunan/16) had cross-reactivity to each other, but did not recognize Jiangxi/09, Italy/00 or Ohio/04 (Fig. 5A-D). Mice vaccinated with Jiangxi/09 or Ohio/04 VLPs (Fig. 5E-F) had antibodies with broad range of cross-reactive HAI activity against all the H7 viruses in the panel, except to Italy/00. In contrast, mice vaccinated with Italy/00 VLPs had broad HAI activity against all the viruses in the panel, except against Jiangxi/09 and Ohio/00 (Fig 5G). Mice vaccinated with NY/02 VLPs elicited antibodies with HAI activity against the homologous NY/02 virus, but did not recognize any of the other H7 viruses (Fig. 5H).

Influence of glycosylation site

With regard to the unique antigenic profile of Italy/00, we found that there was a putative glycosylation site at HA_{169} (H7 numbering from our own sequence alignment) (Table 2). Since the location of putative *N* -linked glycosylation was located in antigenic site B, we hypothesized that glycosylation at this location may be responsible for the unique antigenic profile of Italy/00. To test the hypothesis, we introduced a mutation into the HA nucleotide sequence of Anhui/13 (HA A169T) and looked for the change in reactivity elicited antisera by each VLP vaccine (Fig. 6A). Interestingly, the reactivity of VLP expressing the Anhui/13 HA A169T mutation elicited antibodies with a significant decrease in HAI activity against Anhui/13 and Hunan/13, but no change against the other 6 viruses (Fig. 6B).

Discussion

This study investigated the antigenic breadth of selected H7 panel influenza HA proteins. Since most available H7 HA sequences originated from major human infections, the selected H7 panel strains were similar with the list of candidate vaccine viruses (CVVs) from the WHO[9]. There was a high similarity of amino acid sequences in the putative HA antigenic sites (Table 2). In addition, antibodies elicited by these HA antigens had HAI activity to most of these H7 viruses (Fig. 5). It was consistent with previous findings showing that broad cross-reactivity among H7 influenza viruses isolated from both North American and Eurasian countries [11,21].

Unlike other HA subtypes, the level of cross-reactiveness among H7 HA proteins did not follow phylogenetic similarity or geographic origin. Instead, mutations that altered the glycosylation pattern around the receptor binding site (RBS) played a critical role in shaping the antigenic profile. A single amino acid substitution (HA A169T) caused a significant change in the antigenic profile of Anhui/13 H7 HA. The mutations were based on the distinctive antigenic profile of Italy/00 H7 HA. This protein has an N-linked glycosylation site (NATF) at residue 167-170 of the HA molecule (Table 2). The putative location of the$N$ -glycosylation is adjacent to the receptor binding site of the trimeric form of HAs (Figure 6(C)). Spontaneous occurrence of the N-linked glycosylation sites at the same location in H7 HA proteins was previously reported during the H7N1 epidemics in Italy in the early 2000's [22]. The corresponding mutation A149T (A169T by our numbering) was spontaneous and stable during the passage of the H7N1 viruses in turkeys, which suggests that the mutation can naturally occur during circulation in poultry species [22]. There was no significant influence of the glycosylation site on host tropism, however, the potential change in antigenicity was not investigated[22]. The closest finding to our study was a study conducted by Zost that demonstrated a lysine to threonine mutation at residue 170 of H3 HA (corresponding to H7 HA169) resulted in a significant change in the glycosylation pattern at antigenic site B and antigenic mismatch to the parental virus [23]. This was not limited to residue 169, the glycosylation at a separate location (H7 HA 141T), which also naturally occurs, hindered the access of the epitope to neutralizing antibodies [17]. This motif was initially found at seven amino acids upstream to antigenic site A in the A/Netherlands/219/2003 H7 HA [17]. Similar to this study, introduction of the corresponding mutation into the A/Shanghai/2/2013 H7 HA (identical HA sequence of Anhui/13) decreased the binding of specific monoclonal antibodies and facilitated HA-mediated entry of the virus[17]. Therefore, H7 HA vaccine strategies should aim to cover such variants to prevent severe vaccine mismatches.

Since the human challenge study conducted in the 1970s, the 1:40 HAI titer has been used to predict vaccine effectiveness when an appropriate challenge study is not plausible, such as the annual flu vaccine approval process. [24-26]. While the 1:40 1:40 HAI titer cut-off is sufficient to provide a rough prediction, the specificity of this prediction can be improved by increasing the HAI titer cut-off[25,27]. This is particularly true for subjects with higher revaccination risks, such as the elderly population[25,27]. Also, it appears that the HAI titer cut-off needs to be optimized based on the format of testing antigen, as was observed when measuring the HAI titers by H7 VLPs that tend provide higher HAI titers in the HAI assay than assays using live viruses (Fig. 3). Thus, we applied ROC analysis to optimize the H7 VLP HAI titer cut-off to predict protection of antibodies elicited by H7 HA vaccinations [27]. The adjusted cut-off, 1:80-160 HAI unit, was more useful to predict protection against weight loss following Anhui/13 challenge than the 1:40 HAI titer.

Serum HAI titer only reflects the protection mediated by the receptor blocking antibodies. Influenza virus vaccines confer protection via diverse mechanisms, such as non-HAI antibodies or CD8+ cytotoxic T cells[11,28]. Lung viral clearance is not mediated by HAI antibody response, but rather other mechanisms that recognize whole HA amino acid epitopes, such as CD8 T cell responses.[29]. Serum HAI antibodies are the only proven mechanism that correlates with blocking viral infection [30]. Until clear correlates of protection by non-HAI antibodies or cell-mediated immune responses become available, the serum HAI titer will remain the most reliable indicator to evaluate influenza vaccine effectiveness.

In conclusion, the data presented in this study demonstrated that the cross reactive antibodies are elicited among H7 HA proteins, but the HA sequences are not correlated with the phylogenetic proximity or geographic orientation of the influenza HA antigens. Key amino acid mutations at putative antigenic sites in the H7 HA proteins are important for both elicitation of broadly-reactive antibodies, but also the binding these antibodies to HA specific epitopes. Future studies will focus on developing vaccines to cover all known H7Nx influenza virus strains and future variants with key mutations.

Acknowledgements

The authors would like to thank Amanada Skarlupka for technical assistance. Influenza viruses were provided by BEI resources (Manassas, VA, USA) and by Dr. Mark Tompkins (Athens, GA, USA) The authors would also like to thank the University of Georgia Animal Resource staff, technicians, and veterinarians for animal

care and the staff of the Animal Health Research Center (AHRC) Biosafety Level 3 laboratories for providing biosafety and animal care. Also, the authors thank the members of the CVI protein production core, Jeffrey Ecker, Spencer Pierce, and Ethan Cooper, for providing technical assistance in purifying the recombinant proteins.

Author Contributions

Hyesun Jang - Conceptualization, Formal analysis, Methodology, Writing

Ted M. Ross – Conceptualization, Funding acquisition, Methodology, Writing/Editing

Competing Interests

There is no competing interested in this study.

Funding Information

This work was funded, in part, by the U.S. NIH/NIAID Collaborative Influenza Vaccine Innovation Centers (CIVICs) contract 75N93019C00052 and by the University of Georgia (UGA) (UGA-001). In addition, TMR is supported by the Georgia Research Alliance as an Eminent Scholar.

References

1. Lam TT, Zhou B, Wang J, et al. Dissemination, divergence and establishment of H7N9 influenza viruses in China. *Nature.*2015;522(7554):102-105.

2. Shi J, Deng G, Ma S, et al. Rapid Evolution of H7N9 Highly Pathogenic Viruses that Emerged in China in 2017. *Cell Host Microbe.*2018;24(4):558-568.e557.

3. Sutton TC. The Pandemic Threat of Emerging H5 and H7 Avian Influenza Viruses. *Viruses.* 2018;10(9):461.

4. Yu D, Xiang G, Zhu W, et al. The re-emergence of highly pathogenic avian influenza H7N9 viruses in humans in mainland China, 2019.*Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin.*2019;24(21):1900273.

5. Hatta M, Zhong G, Gao Y, et al. Characterization of a Feline Influenza A(H7N2) Virus. *Emerg Infect Dis.* 2018;24(1):75-86.

6. Belser JA, Blixt O, Chen L-M, et al. Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. *Proceedings of the National Academy of Sciences.* 2008;105(21):7558.

7. Belser JA, Pulit-Penaloza JA, Sun X, et al. A Novel A(H7N2) Influenza Virus Isolated from a Veterinarian Caring for Cats in a New York City Animal Shelter Causes Mild Disease and Transmits Poorly in the Ferret Model. *Journal of virology.* 2017;91(15):e00672-00617.

8. Zoonotic influenza viruses: antigenic and genetic characteristics and development of candidate vaccine viruses for pandemic preparedness.*Releve epidemiologique hebdomadaire.* 2017;92(42):633-647.

9. Dong J, Chen P, Wang Y, et al. Evaluation of the immune response of a H7N9 candidate vaccine virus derived from the fifth wave A/Guangdong/17SF003/2016. *Antiviral research.* 2020;177:104776.

10. Krammer F, Albrecht RA, Tan GS, et al. Divergent H7 Immunogens Offer Protection from H7N9 Virus Challenge. *Journal of virology.*2014;88(8):3976.

11. Stadlbauer D, Rajabhathor A, Amanat F, et al. Vaccination with a Recombinant H7 Hemagglutinin-Based Influenza Virus Vaccine Induces Broadly Reactive Antibodies in Humans. *mSphere.*2017;2(6):e00502-00517.

12. Guo L, Wang D, Zhou H, et al. Cross-reactivity between avian influenza A (H7N9) virus and divergent H7 subtypic- and heterosubtypic influenza A viruses. *Scientific reports.* 2016;6:22045-22045.

13. Carter DM, Darby CA, Lefoley BC, et al. Design and Characterization of a Computationally Optimized Broadly Reactive Hemagglutinin Vaccine for H1N1 Influenza Viruses. *Journal of virology.* 2016;90(9):4720.

14. Huang Y, Owino SO, Crevar CJ, Carter DM, Ross TM. N-Linked Glycans and K147 Residue on Hemagglutinin Synergize To Elicit Broadly Reactive H1N1 Influenza Virus Antibodies. *Journal of virology.*2020;94(6):e01432-01419.

15. Matrosovich M, Matrosovich T, Garten W, Klenk HD. New low-viscosity overlay medium for viral plaque assays. *Virology journal.*2006;3:63.

16. Unal I. Defining an Optimal Cut-Point Value in ROC Analysis: An Alternative Approach. *Computational and Mathematical Methods in Medicine.* 2017;2017:3762651.

17. Alvarado-Facundo E, Vassell R, Schmeisser F, Weir JP, Weiss CD, Wang W. Glycosylation of Residue 141 of Subtype H7 Influenza A Hemagglutinin (HA) Affects HA-Pseudovirus Infectivity and Sensitivity to Site A Neutralizing Antibodies. *PloS one.* 2016;11(2):e0149149-e0149149.

18. Liu D, Shi W, Shi Y, et al. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. *The Lancet.*2013;381(9881):1926-1932.

19. Gao R, Cao B, Hu Y, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. *The New England journal of medicine.*2013;368(20):1888-1897.

20. Habibzadeh F, Habibzadeh P, Yadollahie M. On determining the most appropriate test cut-off value: the case of tests with continuous results. *Biochem Med (Zagreb).* 2016;26(3):297-307.

21. Schmeisser F, Vasudevan A, Verma S, et al. Antibodies to antigenic site A of influenza H7 hemagglutinin provide protection against H7N9 challenge. *PloS one.* 2015;10(1):e0117108-e0117108.

22. Iqbal M, Essen SC, Xiao H, Brookes SM, Brown IH, McCauley JW. Selection of variant viruses during replication and transmission of H7N1 viruses in chickens and turkeys. *Virology.* 2012;433(2):282-295.

23. Zost SJ, Parkhouse K, Gumina ME, et al. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. *Proceedings of the National Academy of Sciences.* 2017;114(47):12578.

24. Wood JM, Levandowski RA. The influenza vaccine licensing process. *Vaccine.* 2003;21(16):1786-1788.

25. Coudeville L, Bailleux F, Riche B, Megas F, Andre P, Ecochard R. Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a bayesian random-effects model. *BMC Medical Research Methodology.*2010;10(1):18.

26. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond).*1972;70(4):767-777.

27. Benoit A, Beran J, Devaster J-M, et al. Hemagglutination Inhibition Antibody Titers as a Correlate of Protection Against Seasonal A/H3N2 Influenza Disease. *Open Forum Infect Dis.*2015;2(2):ofv067-ofv067.

28. Tan GS, Leon PE, Albrecht RA, et al. Broadly-Reactive Neutralizing and Non-neutralizing Antibodies Directed against the H7 Influenza Virus Hemagglutinin Reveal Divergent Mechanisms of Protection. *PLOS Pathogens.* 2016;12(4):e1005578.

29. Schmidt ME, Varga SM. The CD8 T Cell Response to Respiratory Virus Infections. *Frontiers in immunology.* 2018;9:678-678.

30. Gianchecchi E, Torelli A, Montomoli E. The use of cell-mediated immunity for the evaluation of influenza vaccines: an upcoming necessity. *Human vaccines & immunotherapeutics.*2019;15(5):1021-1030.

Figure legend

Figure 1. Frequencies of influenza HA clusters in 2000 and 2018. Total 3633 Influenza HA sequences uploaded in between 2000 and 2018 in GISAID databases were aligned to understand how the H7Nx viruses has evolved. Due to the overwhelming number of Anhui/13-like viruses during Asian H7N9 epidemics, the pie chart analysis was separately conducted on sequences isolated before and after 2013 Asian H7N9 epidemics (A and B). The non-Asian H7N9 sequences isolated after 2013-2018 were further analyzed as a separate pie (C). The aligned sequences were clustered by 3% amino acid similarity and depited into each pie. The viruses to represent each pie were chosen from WHO candidate vaccine viruses.

Figure 2. Study Design C57BL/6 mice designated as H7 panel strains were vaccinated with H7 virus like particles (VLPs) via intramuscular route (3μg/mouse) for two times with a four-week-interval. At week 8, mice were bled to measure serum antibody response, followed by stringent challenge with A/Anhui/1/2013 H7N9 (100 LD_{50}). Mice were monitored for clinical symptoms and weight loss for 14 days. At day 4, three mice were randomly selected from each group to assess viral lung titers.

Figure 3. Anti-Anhui/13 hemagglutination inhibition (HAI) titers Serum samples collected at week 8 tested for the HAI antibody response specific to A/Anhui/1/2013 H7 virus like particles (VLPs) and A/Anhui/1/2013 H7N9 virus (A and B, respectively. Individual titer was plotted and the mean value was presented as bars. Dotted line represents the lower detection limit (10 HAI unit)

Figure 4. Protection against stringent H7N9 challenge C57BL/6 mice (8 mice/group) vaccinated with H7 VLPs at week 0 and 8 were intranasally infected with 10e+5 PFU of the A/Anhui/1/2013 H7N9) virus. Mice were monitored daily for weight loss (A and B) and viral lung titers in selected mice 4 days post challenge (C). Weight loss and lung viral titer was presented as mean± standard deviation (Aand C). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 5. Cross-reactiveness among H7 panel strains The week 8 sera was tested for the cross-reactivity to H7 VLPs expressing HA from all nine panel strains. Individual titer was plotted and the mean value was presented as bars. Grey box indicates the cut-off as the protection surrogate (80-160 HAI unit)

Figure 6. (A) Mutagenesis to Anhui/13 H7 HA Site directed mutation was conducted on plasmid expressing wildtype (WT) Anhui/13 HA. to result in alanine to threonine substitution at HA_{169}. The mutation is expected to result in alanine to threonine substitution at HA169 (H7 numbering) **(B) Change of cross-reactiveness by the mutagenesis** The plasmid with mutation was expressed as virus-like particle (VLP) tested for the reactivity to anti-H7 panel sera. Individual HAI titer was plotted. The box indicates the mean± standard deviation. *p<0.05 **(C) Predictive location of mutation on the HA trimer** The trimeric structure of Anhui/13 H7 HA was generated using the 3D-JIGSAW algorithm, and renderings were performed using MacPyMol. The putative antigenic site B and mutation site (H7 HA169) was shown in blue and red, respectively. Dashed circle indicates receptor binding site.

S1. Determination of HAI cutoff using receptor operating curve (ROC) analysis. The plots of sensitivity% versus false positive rate (100-specificity%) of each cut-off were connected to form the ROC curve (prism). Sensitivity= #of mouse which showed hemagglutination inhibition (HAI) titer [?] cut-off and was protected from the challenge study/ # of all protected mice, Specificity = #of mouse which showed hemagglutination inhibition (HAI) titer < cut-off and unprotected from the challenge study/ # of all unprotected mice, Youden's index = Sensitivity + Specificity -1

Hosted file

`Jang Figure 1.pptx` available at https://authorea.com/users/346877/articles/472693-

hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Figure 2.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Figure 3.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Figure 4.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Figure 5.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Figure 6.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Table 1.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins

Hosted file

Jang Table 2.pptx available at https://authorea.com/users/346877/articles/472693-hemagglutination-inhibition-antibody-landscapes-after-vaccination-with-diverse-h7-hemagglutinin-proteins