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EVALUATION OF ANTIBODY RESPONSE AGAINST G4 AND G1 EURASIAN AVIAN-LIKE H1N1 STRAINS WITH HA VACCINATION IN SWINE.

by
Callie Olson

A Thesis Submitted in Partial Fulfillment
Of the Requirements for the
University Honors Program

Department of Biology
The University of South Dakota
May 2022

The members of the Honors Thesis
Committee appointed to examine the thesis of
Callie Olson find it satisfactory and
recommend that it be accepted.

Dr. Victor Huber, Ph.D.
Associate Professor, Basic Biomedical Sciences
Director of Committee

Dr. Dawn Bragg, Ph.D.
Dean of Student Affairs, SSOM

Beate Wone
Instructor, Biology Department

ABSTRACT

EVALUATION OF ANTIBODY RESPONSE AGAINST G4 AND G1 EURASIAN AVIAN-LIKE H1N1 STRAINS WITH HA VACCINATION IN SWINE.

By: Callie Olson

Director: Victor Huber, PhD

We are investigating the use of the Huber laboratory H1N1 influenza broad-antibody response vaccine product candidates HA-111 and HA-113 against emerging swine H1N1 virus variants *G1* and *G4* that are circulating in China. The question is, what would happen in the event that the *G1* and/or *G4* viruses were to jump from swine to humans and become transmissible between humans? As a subtype of influenza A viruses, H1N1 viruses have pandemic potential due to their ability to infect a variety of hosts other than humans and acquire new gene segments from those other reservoirs. Broad-coverage influenza vaccines are of great interest in research today, as they could have the potential to limit the next pandemic if it were to be a result of an influenza virus. We hypothesize that our HA-111 and HA-113 vaccines will induce broad-coverage as they introduce antibodies that react with recent H1N1 viruses, including *G1* and *G4*. Using the hemagglutination inhibition assay, fetal pig serum sample groups were tested for broad-antibody response against the H1N1 viruses *G1, G4, Cal09/pdm, MI15, GU19, BR18,* and *VIC19.* Analysis of the HAI titers indicated that the HA-113 vaccine candidate shows a significant broad-antibody response against H1N1 viruses, greater than that of the HA-111 vaccine candidate.

Key Words: influenza, propagation, titer, hemagglutination, pandemic

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I. Introduction

Influenza viruses are ever-changing viruses that circulate among humans and animals. There are four types of influenza known as influenza A, influenza B, influenza C, and influenza D. Humans are primarily impacted by the A and B types of influenza, as they are known to cause epidemics nearly every winter season in the United States. Influenza C viruses can infect humans, but typically only cause mild illness and do not qualify as epidemics in their spreading abilities. As for influenza D viruses, they are not known to infect humans, but rather infect cattle. Although influenza B viruses have the ability to cause seasonal epidemics, the only influenza viruses with pandemic potential in humans are influenza A viruses. Additionally, Influenza A viruses are able to infect a variety of hosts aside from humans, including swine and birds (CDC, 2021). This is what gives them their pandemic potential (ability to acquire new segments from different species).

Influenza A viruses can be classified further into subtypes based on their two surface proteins known as hemagglutinin (H) and neuraminidase (N). A very well-known influenza subtype is H1N1, which caused the influenza pandemic of 2009. More specifically, the *H1N1 Cal09/pdm* virus caused the influenza pandemic of 2009 (CDC, 2021). The *Cal09/pdm* virus is a combination of viruses from pigs, birds, and humans that causes disease in humans. Nowadays, the *H1N1 Cal09/pdm* virus is one of the many strains that cause seasonal influenza epidemics and is one of the target viruses for the annual influenza vaccine administered across the country (Mayo Clinic Staff, 2021). As viruses exist and are passed between individuals, they adapt and undergo genetic changes which result in new strains of the virus (CDC, 2021). Once a virus undergoes enough genetic change and antigenic change to be unrecognizable by antibodies against the

original strain, a new clade is formed and there is a chance that cross-immunity is no longer present (CDC, 2021). This poses an issue for vaccine coverage, which is why development of broad-coverage influenza vaccines against diverging strains of the virus are of interest.

The influenza vaccines studied in the Huber lab were developed for both swine and humans (McCormick et al., 2015). During the *Cal09/pdm* pandemic, it was shown that controlling swine influenza infection is critical to preventing the rise of human influenza viruses (Li et al., 2020). This is especially true due to the possibility of novel viruses evolving within swine populations, resulting in viruses with amplified transmissibility and virulence among humans as observed in the 2009 pandemic. It is for this reason that vaccines that limit swine influenza circulation and prevent interspecies transmission are of great interest (McCormick et al., 2015). Thus, the HA-111 and HA-113 vaccine candidate products were developed for broad antibody response against H1N1 viruses using shuffled chimeric HA gene sequences from the *TN09, OH07, NJ76, and IA06* H1N1 viruses (McCormick et al., 2015) (Li et al., 2020). These HA-based vaccine products were the products used in this study.

As outlined in Sun et al. (2020), there has been systematic surveillance of swine influenza in China in order to provide time to prepare for potential upcoming pandemics in humans. In the surveillance completed between the years 2011 and 2018, the genotype 1 (*G1*) and genotype 4 (*G4*) Eurasian avian-like (EA) H1N1 viruses were identified as bearing resemblance to the 2009 pandemic virus and as being the predominant influenza strains present in the swine population. *G1* viruses were the predominant viruses circulating in both northern and southern China at the beginning of the surveillance

between 2011 and 2013. In 2013, the *G4* virus emerged in southern China. It was found that the *G4* virus prevalence has increased dramatically since 2016 and is the predominant genotype detected in pigs in at least 10 provinces of China since then. This is a concern, as the widespread *G4* infection among swine increases the chances of human exposure to the virus, and thus a greater chance of the virus mutating to be able to jump from swine to human. In studying the *G1* and *G4* viruses further, it was found that the *G4* EA H1N1 viruses are more infectious than the *G1* viruses (Sun et al., 2020). Additionally, the Sun et al. (2020) study concluded that the *G4* viruses were able to efficiently replicate inside human airway epithelial cells, indicating the viruses' ability to grow and cause illness in humans if infected (Sun et al., 2020). These newly circulating strains of H1N1 are cause for questioning what would happen in the event that the *G1* and/or *G4* viruses become human-to-human transmissible and begin the next pandemic.

My thesis will investigate the potential of the HA-111 and HA-113 vaccine product candidates in producing immunity against the *G1* and *G4* viruses. Day post-challenge 5 (DPC5) fetal pig serum samples were taken and tested against various H1N1 viruses, including the *G1, G4, and Cal09/pdm* viruses. Prior to serum samples being extracted, these pigs were administered one of four treatments: HA-111, HA-113, PBS, or Vector. Using the hemagglutination inhibition assay, serum samples were tested for existing immunity against the H1N1 viruses. The results could potentially act as preliminary identification of a pre-pandemic vaccine product to be administered if the *G1* and/or *G4* viruses were to mutate to jump from swine to human and transmit between humans.

II. Materials & Methods

Plasmid Gene Transformation & Propagation of Bacteria

G1 and G4 hemagglutinin (HA) genes (Sun et al., 2020) were created within individual plasmids (Twist Bioscience, San Francisco, CA) with restriction sites (BsmBI) that were used to shuttle HA genes into the pHW2000 plasmid, as described by Shepardson et al. (2019). 5 µL of pHW2000-ligated plasmid insert was added to a 50 µL aliquot of DH5a cells. The tube was incubated on ice for 30 minutes, flicking every ten minutes. The mixture was then heat shocked at 37ºC for 25 seconds by holding in 37ºC water bath, followed by chilling on ice for at least 2 minutes. 950 µL of LB (no AMP) was added to the DH5a and plasmid insert tube over open flame and incubated at 37ºC shaking (225 rpm) for at least one hour. 100 µL of bacterial suspension was added to LB + Amp plate (supe) and the remaining suspension was centrifuged at maximum speed for 30 seconds. The supernatant was removed, and pellet was resuspended completely in 150 µL LB + Amp. The suspension was plated on LB + Amp (pellet), and both plates were incubated overnight at 37ºC. Post incubation, the plates were wrapped in ParaFilm and stored at 4ºC the next morning. In the afternoon, colonies were picked with a loop and swirled into bacterial culture tubes containing 5 mL LB + Amp. Controls included pHW2000 alone and PR8 HA in 10 mL LB + Amp to compare plasmid migration on gel. The culture tubes were incubated overnight at 37ºC with shaking (225 rpm).

Samples with growth in tubes were then mini-prepped. 1.5 mL of cultures were centrifuged for 5 minutes at maximum speed, supernatant discarded. The pellet was resuspended in 250 µL P1 by pipetting, followed by adding 250 µL P2 & mixing, and adding 350 µL N3 & mixing. Samples were then centrifuged at maximum speed for 10

minutes and supernatant was added to new columns. The columns were centrifuged 1 minute at maximum speed and discarded flowthrough. We added 750 µL PE buffer, centrifuged 60 seconds, and discarded flow through. Columns centrifuged another 60 seconds to dry and placed in clear tube. 50 µL RNase-free water was added to column, let sit 1 minute, then centrifuged 60 seconds. Following Mini-Prep, agarose gel was poured and ran with samples to confirm successful ligation.

MDCK Cell Line Passaging

This procedure was performed under the hood using aseptic techniques. Two trypsin aliquots were thawed in a water bath. Meanwhile, the MDCK cell flask was removed from the 37°C and 5% CO_2 incubator and the media inside was dumped into a beaker. The 150 cm^2 flask was then washed with 50 mL sterile 1X PBS. Trypsin aliquots were removed from water bath and combined with 9 mL of PBS in each to bring the volume up to 10 mL per aliquot tube. The trypsin was pipetted into the flask, followed by the flasks being incubated at 37°C and 5% CO_2 for 30 minutes to 1 hour. After incubation, the trypsin containing cells were pipetted off and transferred to a 50 mL corning tube. Corning tubes were centrifuged for 3 minutes at 2,500 rpm to pellet the MDCK cells, followed by discarding the remaining trypsin into beaker. The MDCK pellet was resuspended in MDCK growth media by pipetting 10 mL up and down until no clumps were present. A new flask was labeled with cell line, date, and initials, and 38 mL of MDCK growth media + 2 mL resuspended cells were added to the flask. The flask was placed in the incubator to allow for cell growth for 72 hours at 37°C and 5% CO_2 before passaging again to allow growth but prevent overgrowth.

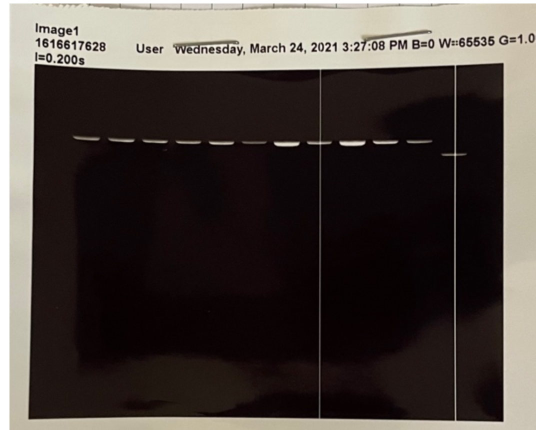

Figure 1

Figure 1: G1 HA Gel Results. Columns 1-10 from left to right contain mini-prepped G1 HA samples grown on LB + Amp plates. Column 11 contains PR8 HA control and Column 12 contains PHW2000 control. HA in PR8 should migrate at about 5,000 bp while pHW2000 should migrate at 3,000 bp.

Reverse Genetics, Virus Rescue, and Propagation

Reverse genetics, virus rescue, and propagation were completed to generate influenza virus from plasmids. This procedure spanned 6 days and was completed under the hood using aseptic technique. On day 1, a co-culture 6-well plate was seeded containing 293T and MDCK cells. To begin splitting each cell flask the cells were washed once with 10 mL of 1X sterile PBS and 10 mL trypsin-EDTA was added to confluent cells in a 75 cm^2 flask. Cells were spun in 15 mL conical tubes, supernatant was discarded, and cells were resuspended in 10 mL OptiMEM I-ABX. A 1:10 dilution of the cells was made in a microcentrifuge tube by adding 400 µL of OptiMEM I-ABX to 100 µL of resuspended cells and 500 µL of trypan blue. The tube was mixed well and 10 µL was added to hemocytometer for cell counting to determine the cells per mL concentration in the conical tubes. Cells were diluted to a final cell count of 7.2 X 10^5 for MDCK's in OptiMEM I-ABX and 7.2 X 10^6 for 293T's in OptiMEM I-ABX to bring a

volume of 19 mL total with OptiMEM I-ABX. We added cells to 6 well plate, 3 mL per well and incubated at 37ºC for 16-18 hours.

On the second day, we added 1 µg of each of the eight segments of reverse genetics system plasmids (maxi-prepped according to Qiagen instructions) to a blue Eppendorf tube. The volume was brought up to 182 µL using OptiMEM + ABX and mix by vortexing. We then added 18 µL of PEI (warm transit at 37ºC for about 5 minutes to make homogenous) to the blue Eppendorf tube. This was mixed well by vortexing and incubated for 20 minutes at room temperature. We removed media from MDCK:293T co-culture monolayers (50-70% confluent) using an aspirator from the side of each well and added 800 µL of fresh OptiMEM I-ABX, followed by 200 µL of the above transfection mixture dropwise to different areas of each well, prior to incubation for 6 hours at 37ºC. After this (on the third day), the transfection mixture was slowly pipetted off the cells and 1 mL of OptiMEM + ABX was added to each cell, followed by incubation for 24 hours at 37ºC.

<table>
<tr><td>A1
G4 HA
Master Mix Reverse Genetics
OptiMEM
PEI</td><td>A2
OptiMEM</td><td>A3
PR8 HA
Master Mix Reverse Genetics
OptiMEM
PEI</td></tr>
<tr><td>B1
G4 HA
Master Mix Reverse Genetics
OptiMEM
PEI</td><td>B2
OptiMEM
PEI</td><td>B3
PR8 HA
Master Mix Reverse Genetics
OptiMEM
PEI</td></tr>
</table>

Figure 2: MDCK:293T co-culture 6-well plate representation for virus rescue G4 in cells A1, B1. Cells A2, B2 acted as negative control and Cells A3, B3 acted as positive control.

On the fourth day, 1 mL OptiMEM + ABX supplemented with 1 µg/mL TPCK-trypsin was added to the 1 mL of media already in the wells (total volume 2 mL per well) and incubated for an additional 42 hours at 37ºC. On the fifth day, MDCK cells from the

flask were split into a separate 24-well plate as described above and incubated overnight at 37ºC to achieve 80-95% confluency. On day 6, virus media was collected into 2 mL tubes and centrifuged 5000 rpm for 5 minutes. After centrifuging, the supernatant was transferred into fresh 2 mL tubes. The samples were then tested for virus by $TCID_{50}$.

In addition to the $TCID_{50}$, undiluted supernatant from the above assay was used to inoculate the MDCK cells seeded in a 24-well plate on day 5. The media on the MDCK monolayers prepared was removed and the cells were washed twice with 500 µL sterile PBS per well. 100 µL of the desired virus was added to the confluent MDCK monolayers (1 well of 6-well plate corresponds with 1 column of 24-well plate). This was incubated at 37ºC for 1 hour, tapping the plate every 15 minutes. After this, 900 µL MDCK infection media + 1 µg/mL TPCK-trypsin was added to each cell and incubated for 72 hours at 37ºC. Each day, the plate was observed for cytopathic effect (CPE). To confirm presence of virus, 50 µL of supernatant was mixed with 50 µL 0.5% chicken red blood cells (in PBS) in a round-bottom 96-well plate and observed for hemagglutination. Virus media was collected into 2 mL tubes, centrifuged at 5000 rpm for 5 minutes, and the supernatant was transferred into fresh 2 mL tubes for storage in -80ºC.

Viral $TCID_{50}$ for Microneutralization Assay of Influenza

The $TCID_{50}$ of the virus must be determined prior to further assay use to confirm the titer of the virus. This procedure spans three days. On Day 1, MDCK cells are seeded at concentration 3.0×10^5 cells mL^{-1} MDCK growth media. 100 µL of cells were added to each well of a 96-well plate and incubated overnight at 37ºC, 5% CO_2. Moving to day 2, the virus was diluted 1:50 in MDCK infection media. In a sterile 96-well tissue culture

plate, 100 µL of MDCK infection media was added to all wells, excluding those in column 1. Instead, 146 µL of virus (1:50 diluted) was added to column 1. The virus was then serially diluted through the plate by transferring 46 µL through the plate in the infection media, resulting in each well having a final volume of 100 µL. Column 12 was left with 100 µL MDCK infection media alone as a cell control. Four of the rows consisted of the G4 virus, and the remaining four rows consisted of the G1 virus to get an accurate $TCID_{50}$ reading. 100 µL of MDCK infection media was added to each well, resulting in the viruses being diluted in ½ log dilutions beginning at 10^{-2}. The plates were then incubated for 2 hours at 37ºC, 5% CO_2. The cells seeded on day 1 were then washed twice with 200 µL per well 1X sterile PBS. 100 µL per well of the corresponding cell treatment from the virus-dilution plates was then added to the MDCK cell plate and incubated with virus for 37ºC, 5% CO_2. The plates were rinsed once with 200 µL per well MDCK infection media. 100 µL of MDCK infection media containing 2 µg mL^{-1} TPCK-trypsin was added to each well and incubated overnight 18-22 hours at 37ºC, 5% CO_2.

The day 3 procedure began with fixing cells. Infection media was removed from the cells, and the plate was rinsed twice with 125 µL per well 1X PBS. The cells were then fixed with 100 µL ice cold fixative per well for 10 minutes at room temperature. The fixative was removed and the plate was air-dried for 20 minutes. The plate was washed three times with 1X PBS + 0.05% Tween-20 and 100 µL 1º antibody (mouse anti-NP monoclonal antibody pool from Dr. Webster's lab) diluted 1:2000 in blocking buffer was added to each well and incubated for 1 hour at room temperature. After incubation, the plate was washed four times with 1X PBS + 0.05% Tween-20. Then, 100 µL per well 2º antibody (goat anti-mouse IgG-HRP) diluted 1:2000 in blocking buffer was added and

incubated 1 hour at room temperature. The plate was washed six times with 1X PBS + 0.05% Tween-20. 100 μL of substrate was added per well, the plate was allowed to develop for 5-10 minutes, or until the background wells began showing color change. Once this occurred, 100 μL per well stop solution (1N H_2SO_4) was added to each plate. The plate was read at 490 nm and the $TCID_{50}$ was calculated using the method of Reed and Muench.

Sample Serum Preparation

Day post-challenge 5 (DPC5) fetal pig serum samples were receptor-destroying enzyme (RDE) treated prior to testing against virus. One volume of each serum sample was added to three volumes of RDE stock. The samples were then incubated overnight in a 37°C water bath. Following this incubation, three volumes of 2.5% sodium citrate solution were added to the samples. These solutions were then incubated in a 56°C water bath for 30 minutes. After the incubations, 3 volumes of 1x sterile PBS were added to each sample, the final dilution being 1:10 of original serum samples. Following the RDE treatment, the samples were pre-cleared using packed chicken RBC to be used in HAI assay. This was done by adding 50 µl of packed RBCs to each treated serum sample and incubating at 4°C for 1hr, mixing every 15 minutes. After this incubation, the samples were spun down at 1000 RCF for 10 minutes. Lastly, the serum samples were removed via pipetting without disturbing the RBC pellets and placed into new Eppendorf tubes.

HAI Assay

A hemagglutination inhibition (HAI) assay was completed using the RDE-treated samples described above against H1N1 viruses *Cal09/pdm*, *G1, G4, GU19, BR18, MI15, and VIC19*. Virus samples were brought to a 1:8 dilution and a titer was done to confirm. In a 96 well round bottom plate, 25 µl PBS was added to columns 2 through 12. Each row corresponded to one serum sample being investigated. Individual 96 well round-bottom plates were used to analyze each virus. 50 µl of each serum stock was put into well 1 of its corresponding row. The sera were serially diluted from columns 1 to 12, transporting 25 µl each time. The last 25 µl to be transferred from column 12 was discarded so that each well would contain only 25 µl of diluted sera. Following this, 25 µl of the 1:8 diluted virus stocks were added to each well of their respective 96 well plates. The plates were then covered in parafilm and incubated at 4°C for 1hr. Following the incubation, 50 µl of 0.5% CRBC stock was pipetted into each well of the plates. Plates were incubated at RT for 30 minutes. The agglutination inhibition titer was then read and recorded for each serum on every plate.

III. Results

HAI Assay Results

The serum samples collected and analyzed via the hemagglutination inhibition (HAI) assay were grouped according to the treatment-group of the pig each sample was taken from. In HAI assays, the target titer indicating significant immune response is 1:40, a 4-fold difference from no response. There were four different treatments given to the pigs depending on their grouping. Those treatments being: HA-111, HA-113, PBS, and Vector. As described in the introduction, HA-111 and HA-113 are HA vaccine candidate

products. PBS and Vector treatment were used as the negative control treatments of the study. Table 1 identifies the groups that each serum sample analyzed belonged to.

Pig Treatment			
HA-111	**HA-113**	**PBS**	**Vector**
51B	57B	54B	64B
52B	58B	69B	65B
53B	59B	72B	66B
55B	60B	73B	67B
56B	61B	74B	68B
70B	62B	75O	
		73O	

Table 1: A table identifying which of the four pig treatment groups each pig serum sample was collected from.

The results of the HAI analysis of the samples against the H1N1 viruses *Cal09/pdm, GU19, BR18, MI15, VIC19, G4, and G1* is divided based upon treatment groups shown above in Table 1. The results for the HA-111-Treated group are illustrated below in Figure 3, with the exact titer values in Table 2 below the figure. Hemagglutination inhibition results that read as a titer of <10 were given a titer of 5 on the graphs for statistical purposes. Very limited hemagglutination inhibition was seen against viruses *GU19, BR18, MI15, and VIC19*, indicating limited immunity. As for *Cal09/pdm, G4, and G1* viruses, more of a response was observed, the largest response being against *G4*. Overall, the response seen from HA-111-treated sera was not present and consistent among all the viruses.

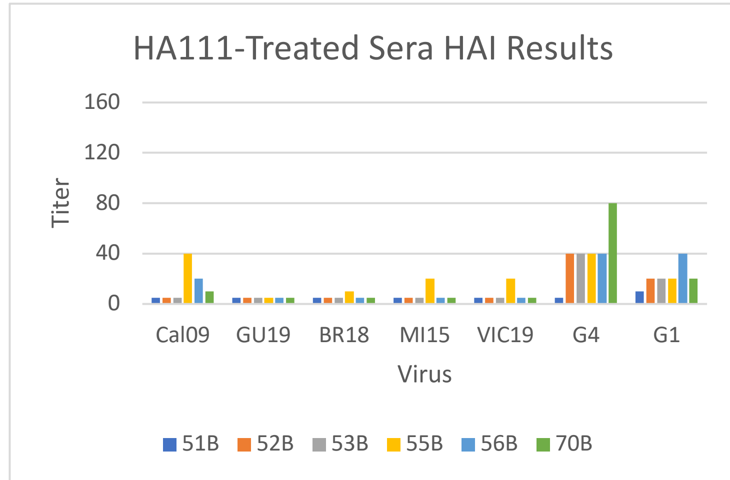

Figure 3: A graph illustrating the HAI assay results for the sera belonging to the HA-111-treated pigs against various viruses. Exact values can be found in Table 2. A titer of 1:40 or greater indicates a significant positive response.

HA-111-Treated Pig Sera HAI Titer							
Sample	**Cal09/pdm**	**GU19**	**BR18**	**MI15**	**VIC19**	**G4**	**G1**
51B	<10	<10	<10	<10	<10	<10	10
52B	<10	<10	<10	<10	<10	40	20
53B	<10	<10	<10	<10	<10	40	20
55B	40	<10	10	20	20	40	20
56B	20	<10	<10	<10	<10	40	40
70B	10	<10	<10	<10	<10	80	20

Table 2: A table showing the HAI assay results for the sera belonging to the HA-111-treated pigs against various viruses.

The hemagglutination inhibition results for the HA-113-treated sera can be found below in Figure 4 and Table 3. Hemagglutination inhibition results that read as a titer of <10 are were given a titer of 5 on the graphs for statistical purposes. Similar to the HA-111-treated sera, there was a very limited response to the *GU19 and VIC19* viruses for HA-113-treated sera. As for the *BR18 and MI15* viruses, the HA-113-treated sera produced a much greater response than that of the HA-111. Additionally, more consistent, and higher titers were also observed between the serum samples when tested against the *Cal09/pdm, G4, and G1* viruses with this vaccine candidate product use. One serum sample was not analyzed against the *G1* virus due to the quantity of the serum sample available for testing.

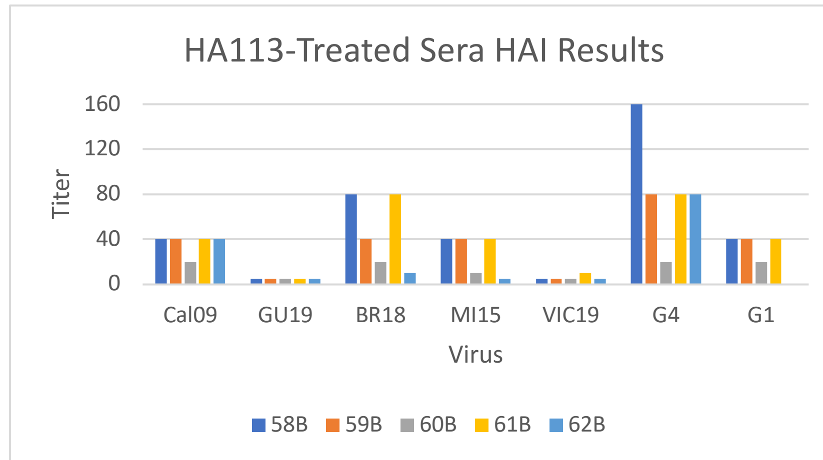

Figure 4: A graph illustrating the HAI assay results for the sera belonging to the HA-113-treated pigs against various viruses. Exact values can be found in Table 3. A titer of 1:40 or greater indicates a significant positive response.

HA-113-Treated Pig Sera HAI Titer							
Sample	**Cal09/pdm**	**GU19**	**BR18**	**MI15**	**VIC19**	**G4**	**G1**
58B	40	<10	80	40	<10	160	40
59B	40	<10	40	40	<10	80	40
60B	20	<10	20	10	<10	20	20
61B	40	<10	80	40	10	80	40
62B	40	<10	10	<10	<10	80	X

Table 3: A table showing the HAI assay results for the sera belonging to the HA-113-treated pigs against various viruses. The "X" indicates that the serum sample was not analyzed for that virus due to quantity of sample available.

The hemagglutination inhibition results for the PBS-treated sera can be observed below in Figure 5 and Table 4. Hemagglutination inhibition results that read as a titer of <10 were given a titer of 5 on the graphs for statistical purposes. As expected, all serum tested against the *Cal09/pdm, GU19, BR18, MI15, VIC19, and G4* viruses yielded titers of <10. One serum sample was not analyzed against the *G1* virus due to the quantity of the serum sample available for testing. The remainder of the PBS-treated serum yielded a very minimal titer of 10 against the *G1* virus. As stated earlier, in order to classify the response as a significant positive, there must be a 4-fold titer difference (i.e. the titer of 10 does not define a positive result). Overall, no solid immunity was seen in the PBS-treated serum.

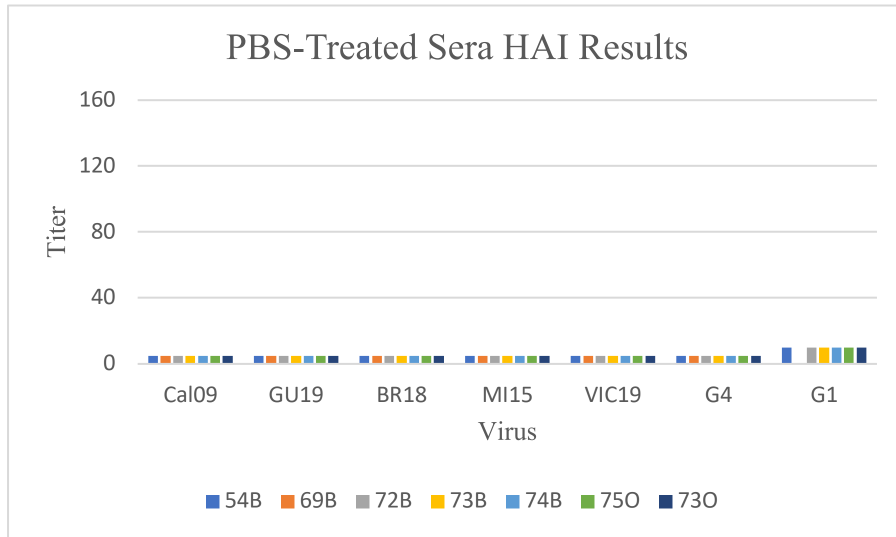

Figure 5: A graph illustrating the HAI assay results for the sera belonging to the PBS-treated pigs against various viruses. Exact values can be found in Table 4. A titer of 1:40 or greater indicates a significant positive response.

PBS-Treated Pig Sera HAI Titer							
Sample	**Cal/09**	**GU19**	**BR18**	**MI15**	**VIC19**	**G4**	**G1**
54B	<10	<10	<10	<10	<10	<10	10
69B	<10	<10	<10	<10	<10	<10	X
72B	<10	<10	<10	<10	<10	<10	10
73B	<10	<10	<10	<10	<10	<10	10
74B	<10	<10	<10	<10	<10	<10	10
75O	<10	<10	<10	<10	<10	<10	10
73O	<10	<10	<10	<10	<10	<10	10

Table 4: A table showing the HAI assay results for the sera belonging to the PBS-treated pigs against various viruses. The "X" indicates that the serum sample was not analyzed for that virus due to quantity of sample available.

Sera taken from pigs treated with vector make up the final group tested in this study. The results of hemagglutination inhibition can be found in Figure 6 and Table 5. The results of the vector-treated sera group appear identical to that of the PBS-treated serum group. All serum titers against *Cal09, GU19, BR18, MI15, VIC19, and G4* were read as <10. As for the *G1* virus, the titers for all samples were read as 10. In order to classify the response as a significant positive, there must be a 4-fold titer difference (i.e. the titer of 10 does not define a positive result).

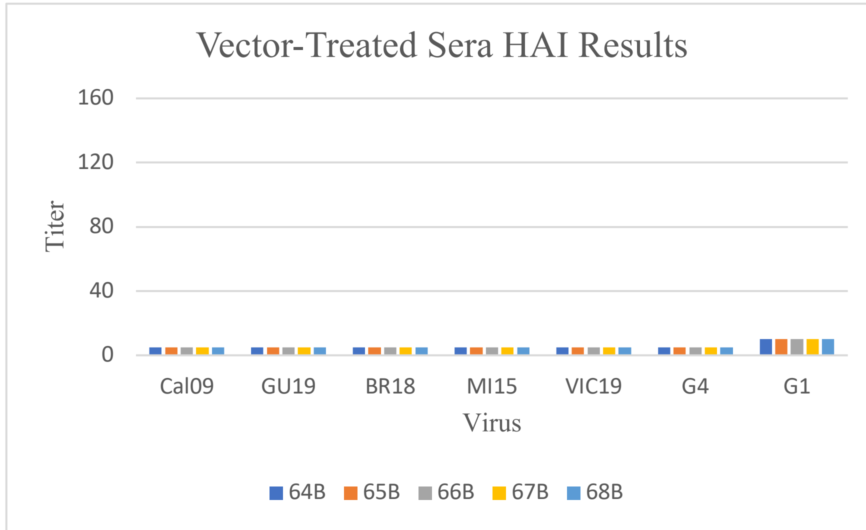

Figure 6: A graph illustrating the HAI assay results for the sera belonging to the vector-treated pigs against various viruses. Exact values can be found in Table 5. A titer of 1:40 or greater indicates a significant positive response.

Vector-Treated Pig Sera HAI Titer							
Sample	**Cal/09**	**GU19**	**BR18**	**MI15**	**VIC19**	**G4**	**G1**
64B	<10	<10	<10	<10	<10	<10	10
65B	<10	<10	<10	<10	<10	<10	10
66B	<10	<10	<10	<10	<10	<10	10
67B	<10	<10	<10	<10	<10	<10	10
68B	<10	<10	<10	<10	<10	<10	10

Table 5: A table showing the HAI assay results for the sera belonging to the vector-treated pigs against various viruses.

TCID50 Readings G1, G4

After stop solution was added to the $TCID_{50}$ plate, the plate was read at 490 nm. A blank reading was also completed at 490 nm to ensure the machine was zeroed correctly prior to the plate reading. The results of the plate reading can be viewed in Figure 7. Rows A-D consist of the *G1* virus while rows E-H consist of the *G4* virus. These numbers are used in determining the $TCID_{50}$ values of the viruses with the Reed & Muench Method as seen and described below.

different numbers because stop added different times

490

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2.896	2.227	1.051	1.223	0.682	0.928	0.633	0.408	0.415	0.336	0.377	0.434	490
B	2.921	2.471	1.940	0.606	0.386	0.361	0.323	0.354	0.384	0.370	0.345	0.369	490
C	3.128	2.279	1.396	0.583	0.449	0.372	0.347	0.308	0.341	0.338	0.349	0.332	490
D	3.075	2.724	1.011	0.829	0.647	0.341	0.341	0.328	0.339	0.355	0.353	0.328	490
E	3.219	3.146	3.147	2.974	2.941	2.704	1.591	0.640	0.510	0.395	0.255	0.252	490
F	3.189	2.961	3.089	3.072	2.724	2.587	1.833	1.431	0.397	0.301	0.311	0.274	490
G	3.118	3.051	3.138	2.976	2.852	2.753	2.251	0.996	0.877	0.293	0.333	0.378	490
H	3.270	3.089	3.162	3.036	2.700	2.529	2.446	0.902	0.815	0.336	0.301	0.288	490
	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7		

.656

.576

Figure 7: A table representing the results of the plate-reader at the end of the $TCID_{50}$ protocol to be used in the Reed & Muench Calculation for $TCID_{50}$ value. Rows A-D contained the *G1* virus, while rows E-H contained the *G4* virus.

The Reed & Muench Method begins with finding the reading of the blank cell. In this case, both D12 and H12 were the blank cells, as the stop solution was added to the top and bottom halves of the plate at different times. Those numbers are then doubled to give the values of .656 for the top half and .576 for the bottom half of the plate. From here, any number in the plate higher than the doubled blank value is considered positive and any number below that value is considered to be negative. The positives and negatives were counted and put into columns “(1) pos” and “(2) neg” of their corresponding tables below: Table 6 and Table 7. The dilution column comes from the dilution of the viruses into half-log differences during the $TCID_{50}$ process. Columns “(3) pos” and “(4) neg” were then filled in by adding together the total number of positives and negatives remaining at that level of dilution. After this, column “(5) ratio” consists of the ratio of positives when the values in “(3) pos” and “(4) neg” are added together, and “(6) % pos” is the percentage of positives associated with the ratio in column 5.

G1 TCID Reed and Muench						
dilution	(1) pos	(2) neg	(3) pos	(4) neg	(5) ratio	(6) %pos
2	4	0	16	0	16/16	100
2.5	4	0	12	0	12/12	100
3	4	0	8	0	8/8	100
****3.5**	2	2	4	2	4/6	66.7
4	1	3	2	5	2/7	28.6
4.5	1	3	1	8	1/8	12.5
5	0	4	0	12	0/12	0
5.5	0	4	0	16	0/16	0

Table 6: Reed and Muench calculation table for *G1* virus for determining $TCID_{50}$ value.

G4 TCID Reed and Muench						
dilution	(1) pos	(2) neg	(3) pos	(4) neg	(5) ratio	(6) %pos
2	4	0	34	0	34/34	100
2.5	4	0	30	0	30/30	100
3	4	0	26	0	26/26	100
3.5	4	0	22	0	22/22	100
4	4	0	18	0	18/18	100
4.5	4	0	14	0	14/14	100
5	4	0	10	0	10/10	100
5.5	4	0	6	0	6/6	100
*****6**	2	2	2	2	2/4	50
6.5	0	4	0	6	0/6	0
7	0	4	0	10	0/10	0

Table 7: Reed and Muench calculation table for *G4* virus for determining $TCID_{50}$ value.

The values in these tables were then used to further calculate and find the $TCID_{50}$/mL values of each virus. The equation associated with the tables to find the $TCID_{50}$ value is $\frac{(\%pos>50)-50}{(\%pos>50)-(\%pos<50)} \times 0.05$. For the *G1* virus the final calculations were as follows:

$$\frac{66.7-50}{66.7-28.6} \times 0.05 = \frac{16.7}{38.1} \times 0.05 = .219 \quad 10^{3.5} + .219 = 10^{3.719}/100\mu L = 10^{4.719} \text{ TCID}_{50}/mL$$

After our final calculation, the determined $TCID_{50}$/mL value of our *G1* virus was $10^{4.719}$ $TCID_{50}$/mL. As for the *G4* virus, the $TCID_{50}$ calculations were as follows:

$$\frac{50-50}{50-0} \times 0.05 = 0 \qquad 10^{6} + 0 = 10^{6}/100\mu L = 10^{7} \text{ TCID}_{50}/mL$$

After the final calculation of the Reed and Muench Method, the determined $TCID_{50}$/mL value of the *G4* virus is 10^{7} $TCID_{50}$/mL.

IV. Discussion

There were significant positive HAI results against the *G1* and *G4* H1N1 viruses in the HA-111-treated and HA-113-treated fetal pig serum groups. Looking more closely at the results for the HA-111-treated samples, the majority of the serum titers against the *G1* virus did not qualify as significant positive results (4-fold titer increase), with only one sample having a titer of 1:40. As for the *G4* virus results with the HA-111-treated sera, the majority of those samples did have a significant positive result, with 4/6 samples at 1:40, 1/6 samples at 1:80, and 1/6 samples reading as negative (<10 titer). Moving on to the HA-113-treated serum results, the majority of the HAI titers against *G1* were read as 1:40, indicating a significant positive reading. When comparing the HA-111 serum group with the HA-113 serum group's HAI titers against the *G1* virus it is clear that the HA-113 group had a greater immune response. The 4/5 of the HA-113 group HAI's resulted in a significantly positive titer when tested against the *G4* virus. These positive titers ranged from 1:80 to 1:160, meaning the HA-113 group had a greater immune response than the HA-111 group did against the *G4* virus.

HAI assays were also conducted with the *GU19, VIC19, Cal09/pdm, MI15,* and *BR18* in order to test how well the HA-111 and HA-113 vaccines protect against more recent H1N1 viruses. The HA-111 and HA-113 vaccines were created using *Cal09/pdm* and were not updated after that point. The HA-111-treated sera produced very little response in the HAI assays against these viruses. Only one significantly positive titer was observed against *Cal09/pdm*. As for the HA-113-treated group, more positive titers were read against multiple viruses. The *Cal09/pdm*, *MI15,* and *BR18* viruses produced significant titers in a majority of the HA-113 group. The maximum titer observed for

Cal09/pdm and *MI15* was 1:40. As for the *BR18* results with the HA-113-treated sera, the maximum HAI titer observed was 1:80.

Overall, the HA-113-treated fetal pig serum produced more significantly positive HAI titers than the HA-111-treated fetal pig serum across the different H1N1 viruses until at least the *BR18* virus. The *BR18* virus circulated about 9 years after the last time the vaccines were updated. As observed through the HAI results with *GU19* and *VIC19,* 2019 appears to be the point where a new vaccine construct is needed. The antibodies did still react with the *G1* and *G4* viruses. The broad coverage of these vaccine products, especially that of the HA-113 vaccine candidate shows the potential of these products to protect against an H1N1 influenza pandemic if one should arise from the *G1* or *G4* swine lineages. By providing coverage against several H1N1 viruses with history of or potential for causing pandemics in this study, it would be hard to ignore the fact that these vaccines could theoretically serve as an early pre-pandemic vaccine if the *G1* and *G4* viruses were to jump from pig-to-human and transmit human-to-human. If these vaccine products were further developed and administered early enough in the beginning stages of human-to-human transmission of the viruses, the pandemic potential of the *G1* and *G4* viruses could be limited.

Broad-antibody response influenza vaccines used in the context of pandemic prevention are especially important when discussed in the context of recent pandemics, namely the COVID-19 pandemic. It took months for a vaccine product against COVID-19 to be created, tested, and put into production. This lead to the rise of the pandemic and the infection and death of millions of people. In the context of this study and the *G1* and *G4* viruses, we may have a vaccine that is ready to go into production rather than one that

needs to go through development first. The development of broad-antibody response vaccines for quickly adapting viruses may be an extremely important component of epidemiology in the coming years in order to prevent future pandemics.

V. References

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