CHARACTERIZATION OF A MULTI-SUBTYPE VIRUS-LIKE PARTICLE (VLP) FOR DEVELOPMENT OF PANDEMIC INFLUENZA VACCINE

by

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DEDICATION

I would like to dedicate this thesis to my parents for their unconditional support in everything I do.

ACKNOWLEDGEMENTS

I would like to thank Dr. Peter Pushko for giving me the opportunity to be a part of this research lab and for his outstanding support and guidance in this project. I would also like to thank Dr. Irina Tretyakova and the team at Medigen for helping me to become a better scientist. I also want to thank my committee members Dr. Boyd and Dr. Smith for their guidance.

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ABSTRACT

Influenza viruses present a global health and economic concern. The most common vaccination methods rely on egg-based vaccines that pose allergy risk and lengthy production time. Currently, there are no widely available pandemic vaccines. Virus-like particles (VLP's) offer a safe and efficient approach for preparing pandemic influenza viruses. In this study, a multi-subtype VLP construct containing H5, H7, H9, and H10 surface glycoproteins was analyzed and quantitated for specific HA expression. All four HA proteins were detected in the VLP using specific anti-HA antibodies, hemagglutinin activity of VLPs was confirmed, and individual HA concentrations were quantitated. Next, mice were vaccinated with this multi-subtype VLP and antibody production was observed. Mice vaccinated with multi-subtype VLP showed hemagglutination inhibition (HI) antibodies against all HA antigens except lower reactivity for H5 antigen. Thus, this study provides evidence for the broad immunogenic and protective potential of the multi-subtype VLP against multiple subtypes of influenza virus.

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LIST OF ABBREVIATIONS

AI	avian influenza virus
Bgag	bovine immunodeficiency virus gag protein
BSA	bovine serum albumin
CD4	cluster of differentiation 4
ELISA	enzyme-linked immunosorbent assay
HA	hemagglutinin
HA Assay	hemagglutination assay
HAU	hemagglutination units
HI Assay	hemagglutination inhibition assay
HPAI	high pathogenic avian influenza
LPAI	low pathogenic avian influenza
LTB	heat-labile enterotoxin B
M1	matrix protein 1
NA	neuraminidase
PBS	phosphate-buffered saline
RBC	red blood cells
RDE	receptor destroying enzyme
RNA	ribonucleic acid
Sf9	*Spodotera frugiperda* insect cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
VLP	virus-like particle
6g VLP	multi-subtype VLP prepared by co-expression of six genes

INTRODUCTION

Influenza viruses remain one of most challenging infectious disease obstacles in the world. Influenza viruses pose as a major public health concern and continue to be a serious economic and societal threat. Influenza outbreaks occur in either seasonal or pandemic forms. Seasonal influenza outbreaks occur annually in the fall and winter months. With the exception of individuals who are at greater risk of complications (e.g., young children, the elderly, pregnant women, chronically ill, immune-compromised, or obese individuals), seasonal influenza is not severe in most healthy individuals (Mayo Clinic 2020). Seasonal influenza affects an average of five to twenty percent of the U.S. population each year. During the 2018-2019 flu season alone, an estimated 35.5 million illnesses, 490,600 hospitalizations, and 34,200 deaths resulted from influenza (CDC 2020). Fifty six percent of the hospitalizations occurred in adults age 65 and older and accounted for 75 percent of flu-related deaths. Approximately 46,000 hospitalizations occurred in children and 8,100 deaths occurred in working-age adults (CDC 2020). Total estimated costs for outpatient visits were 3.7 million and emergency department visits were 0.65 million. The average total annual economic burden to U.S. society and the healthcare system was estimated to be at 11.2 billion dollars (Putri et al. 2018).

The general population has partial immunity to seasonal influenza from previous exposures to similar subtypes. In contrast, pandemic influenza outbreaks occur when a completely new subtype emerges, and the general population has little to no pre-existing immunity. Symptoms could be more severe and even healthy individuals could be at greater risk for serious complications. With seasonal influenza, healthcare systems can generally meet public demands with available annual flu vaccines, antiviral medications,

and can withstand the economic and societal impacts. However, with pandemics, healthcare systems could be overwhelmed. The United States keeps a limited supply of pre-pandemic vaccines, however, it is difficult to predict the strain of pandemic-causing virus until after its emergence. Therefore, vaccines would most likely not be available during early stages. Also, current antiviral drugs may not be effective against the pandemic strain (Public Health 2021). Four pandemic outbreaks have been recorded in history: 1918 H1N1 (50 million deaths worldwide), 1957 H2N2 (1.1 million deaths worldwide), 1968 H3N2 (1 million deaths worldwide), and 2009 H1N1pdm09 (150,000-575,000 deaths worldwide) (CDC 2019). Although rare and unpredictable, experts agree another occurrence of influenza pandemic is undeniable, prompting the need for developing pandemic vaccines.

Influenza Virus Structure, Mutations, and the Threat of Pandemics

Influenza viruses are members of the orthomyxovirus family and have a genome consisting of eight negative sense RNA segments that encode 12 viral proteins. The spherical virion is approximately 80-120 nanometers in diameter and has an outer lipid layer composed of surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). HA binds to sialic acid receptors on the host cell surface to induce viral penetration and functions as the main virus antigen. NA protein mainly functions in aiding viral particle release and is the target of antiviral drugs. The ratio of surface HA to NA composition is 4 to 1. Matrix protein M1 beneath the lipid membrane forms a a shell that provides structure and rigidity. These enveloped viruses are categorized into four types: A, B, C, and D. Influenza A and B cause most of human illnesses. Type A also infects birds, swine, horses,

seals, and dogs. Type B primarily infects humans and rarely infects animals. Type C is rarely reported in humans while type D primarily affects cattle and is not known to cause human illnesses. Type A is mainly responsible for the seasonal and pandemic outbreaks and is further categorized based on the composition HA and NA glycoproteins. Currently, 18 HA subtypes and 11 NA subtypes have been identified. There are 198 possible influenza A subtype combinations, however, only 131 subtypes have been detected (CDC 2019).

HA protein consists of a variable globular head domain and a conserved stem, or stalk, domain. The antigenic sites are mostly located on the head domain. Due to the high replication rate and poor proofreading mechanism of RNA polymerase, there is a high mutation rate in the antigenic sites (Arbeitskreis Blut 2009). The mutated viral progeny can proliferate, escape host neutralization, and re-infect humans. The phenomenon is known as antigenic drift, where over time, these small mutations can accumulate and result in an antigenically different virus. This occurrence is what requires re-administration of new vaccines with each new flu season. The segmented genome facilitates reassortment of different influenza strains in a host susceptible to both human and avian influenza viruses (e.g,, pigs) and results in a new subtype that can then infect humans (Sriwilaijaroen and Suzuki 2012). This phenomenon is known as antigenic shift where there is an abrupt change in a virus that results in novel HA and NA combinations that are distinct from existing subtypes. Since most humans have little to no immunity to the new virus, it can lead to pandemic outbreaks. While antigenic drift occurs constantly, antigenic shift is rare.

For a virus to be considered pandemic, the virus must be novel, highly transmittable from human to human, and be able to cause serious illness or death. Avian influenza A viruses are emerging pathogens that pose pandemic potential as increased avian influenza

virus outbreaks have been reported in humans (Philippon et al. 2020). Influenza A circulating among wild aquatic birds such as ducks, geese, and swans are referred to as avian influenza (AI) A viruses. These birds are natural reservoirs of AI viruses. These viruses infect domestic poultry (e.g, chickens) and other bird and animal species. AI are categorized based on the virus' ability to cause disease in chickens in a controlled lab setting. Low pathogenic avian influenza (LPAI) A viruses cause mild to no disease and may not be detected while high pathogenic avian influenza (HPAI) A viruses cause severe illness and have high mortality rate. Normally, AI viruses do not infect humans, however, many isolated cases have been reported. More than 700 cases of infection by an Asian HPAI H5N1 strain have been reported in over 15 countries since 2003. Symptoms range from mild to fatal. There have been 1,565 reported cases of human infection by Asian lineage avian influenza A H7N9 with a 39 percent fatality in confirmed infections. Infection by AI is often due to close contact with infected or dead poultry. The spread of AI viruses from person to person is rare, and the reported transmissions were limited and not sustained. (CDC 2019).

The threat comes from the close proximity of poultry, pigs, and humans. Since human and avian influenza viruses can both infect pigs, it leads to increased likelihood of genetic reassortment and antigenic shift where a new influenza subtype emerges. If this new virus is able to be transmitted easily from person to person, with the general population having little to no immunity to this new virus, a global epidemic could arise. The past 1918, 1957, and 1968 pandemic strains were believed to have resulted from reassortment of both human and avian strains (McFee 2007). Genetic analyses showed a reassortment of avian, human, and swine viruses resulted in the 2009 pandemic (Clinlab Navigator 2021). To aid

in pandemic preparedness, the World Health Organization keeps surveillance of influenza viruses circulating in animals to detect strains that have the highest potential of becoming pandemic (WHO 2020). The main avian influenza subtypes that have the highest predicted potential for becoming pandemic are H5N1, H7N9, H9N2, and H10N8 (Kang et al. 2009).

Vaccines for Influenza

The most effective method of prevention and infection control is vaccination. For seasonal flu, the World Health Organization Global Influenza Surveillance and Response System (GISRS) reviews the results of surveillance, laboratory, and clinical studies from the national influenza centers in over 100 countries and makes recommendations about which specific strains to include in each annual vaccine for the upcoming season (Houser and Subbarao 2015). Currently, the three types of licensed vaccines for seasonal flu are inactivated, live attenuated, and recombinant HA vaccines, with majority of vaccines produced using eggs.

Unlike seasonal vaccines, pandemic vaccines would need to be manufactured and mass-produced after the outbreak. The logistical challenges of producing vaccines from scratch and delivering in mass numbers are immense (DiMenna et al. 2009). The dependence on embryonated eggs can pose a problem if high demands in the event of a pandemic lead to short supply. Egg-based vaccines pose allergy risk and have a lengthy vaccine production timeline of approximately 6 months (Rajaram et al. 2020). Influenza vaccines grown in eggs can adapt and mutate in the eggs. Therefore, the strain produced from the vaccine may not match the circulating strain. Highly pathogenic avian influenza strains like H5N1 are difficult to grow since they cause severe illness and death in chickens destroying the chicken eggs. H3N2 also grows poorly in eggs which further prompts for

developing alternative methods (Grohskopf et al. 2014). Therefore, improving vaccine design and production methods are of upmost importance for proactively preparing for future influenza pandemics.

Recently, there has been progress in using virus-like particles as an alternative approach for vaccine development. Virus-like particles (VLP's) are protein structures that mimic a real virus while lacking the viral genome to replicate and cause infection (Roldao et al. 2010). Commercially available VLP-based vaccines have been developed for human papilloma virus, hepatitis B virus, and malaria (Mohsen et al., 2017). These VLP's are safer alternatives to live attenuated methods since they do not involve the use of live virus. In addition to the high safety feature, as shown in Figure 1, VLP's can present surface glycoproteins in a highly immunogenic form since they mimic the virus' native conformation.

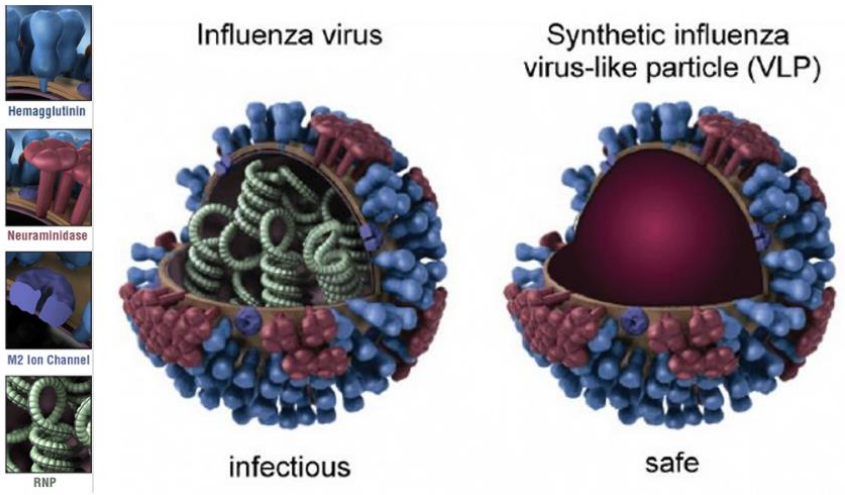

Figure 1. Diagram representing influenza virus structure (left) and an influenza virus-like particle (right). Blue surface glycoproteins represent hemagglutinin and red represents neuraminidase (Adapted from Trowitzsch et al. 2011).

Adjuvant molecules can be directly anchored to the VLP surface to increase immunogenicity. VLP's have been shown to elicit effective antigen presenting cell stimulation, B- and T-cell responses, CD4 T-cell proliferation and cytotoxic T-cell immune responses (Kang et al. 2012*).* These recombinant flu vaccines potentially allow for quicker vaccine production, which is essential for keeping up with the constantly evolving influenza strains. Unlike egg-based vaccines that can mutate in eggs, VLP's are produced with the exact amino acid sequence of the circulating strain. Since VLP's utilize cell-culture methods, they mitigate the hazard of working with live virus and overcome the issue of growing HPAI virus strains. There are several approaches to VLP design for developing influenza vaccines.

One approach involves the conserved HA stalk domain with the aim of developing a universal flu vaccine. One study generated a headless HA VLP and showed effective humoral and cellular responses in mice (Gao et al. 2017*).* Additionally, vaccines targeting the matrix protein M2e have been developed by fusing M2e to a hepatitis B virus core and showed high anti-M2e antibody production (De Filette et al. 2006). Since the antigenic drift and rate of evolution is slower for NA, VLP's expressing NA protein have also been considered. N1 VLP's without HA proteins were able to induce antibody responses. NA VLP immunized mice were protected against lethal dose of H1N1 and H3N2 virus challenge (Quan et al. 2012). Heat-labile enterotoxin B subunit protein (LTB) and Toll-like receptor 5 ligand flagellin (Flic) adjuvants were included in H5N1 VLP structures and resulted in enhanced protective responses in mice (Ren et al. 2018).

Another approach to developing a broadly protective influenza vaccine is the use of multi-subtype VLP's containing different HA proteins in one VLP structure (Figure 2).

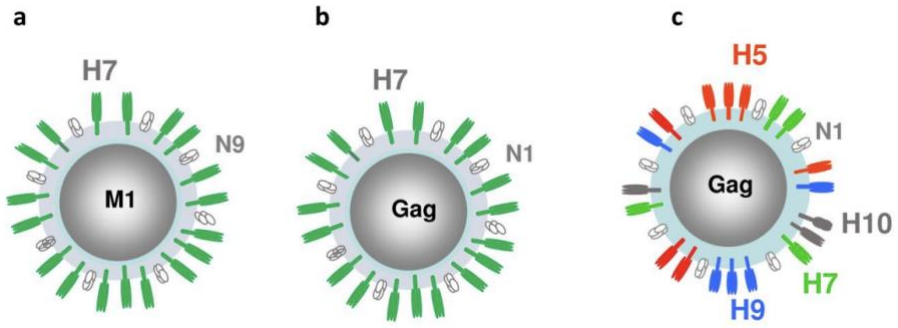

Figure 2. Diagram showing different types of virus-like particles (VLP's). (a) Mono-subtype H7N9 VLP with M1 as scaffold. (b) Chimeric mono-subtype VLP with H7 and N1 with Gag protein as scaffold. (c) Multi-subtype VLP co-localizing N1, Gag, H5, H7, H9, and H10. (Adapted from Pushko and Tretyakova 2020)

Medicago first reported a quadrivalent influenza VLP vaccine generated in *Nicotiana benthamiana* tobacco plant cells in phase II clinical trials with successful cellular and humoral immune responses in subjects (Pillet et al. 2019). VLP's containing H5, H7, and H2 proteins were shown to be immunogenic and offered protection against H5N1, H7N2, and H2N3 viral challenges in ferrets (Pushko et al. 2011). Also, VLP's consisting of H5, H7 and H9 proteins along with NA and M1 proteins were co-expressed in a baculovirus vector and showed antibody induction and efficient protection against H5N1, H7N2, and H9N2 viral challenges in ferrets (Tretyakova et al. 2013). A VLP construct consisting of bovine immunodeficiency virus gag (Bgag) protein in place of M1 protein was generated in *Spodotera frugiperda* (Sf9) insect cells and was able to show HA and NA activities as well as specific antibody production (Tretyakova et al. 2016).

VLP's containing four HA subtype genes (H5, H7, H9, H10) have been shown to elicit protective immunity against H10 influenza in a ferret challenge model (Pushko et al. 2016). This vaccine design method of co-localizing multiple HA subtypes into one VLP can allow for broader protection and can be produced in a single manufacturing process to

decrease production costs. The HA gene sequences in the 2016 study were obtained from A/VietNam/1203/2004 (H5N1 clade 1), A/Shanghai/2/2013(H7N9), A/Hong Kong/33982/2009 (H9N2 clade G1), and A/Jiangxi/IPB13a/2013 (H10N8) viruses. The study used the Bac-to-Bac Baculovirus Expression System by Invitrogen. Using this method, the 6 genes were cloned in tandem fashion into a pFastBac1 vector, then transformed into DH10Bac *Escherichia coli* cells. The successful recombinant DNA, called bacmids, are then transfected into *Sf9* insect cells to produce and amplify VLP proteins. VLP's are harvested and purified using ion exchange chromatography and concentrated by ultracentrifugation. (Thermo Fisher 2021).

To prepare the VLP used in this study, the same baculovirus expression system was used with updated HA strains from A/Sichuan/26221/2014 (H5N6), A/Hong Kong/125/2017 (H7N9), A/Hong Kong/33982/2009 (H9N2), and A/Jiangxi/IPB13a/2013 (H10N8). In this study, we aimed to analyze and quantitate the HA contents of a multi-subtype (abbreviated 6g) VLP construct comprised of H5, H7, H9, H10, N1, and Bgag using SDS-PAGE, western blot, HA and ELISA assays. In addition, we aimed to vaccinate mice with the 6g VLP and observe the production of protective antibodies using hemagglutination inhibition (HI) assay. We expect to observe positive HA bands against specific anti-HA antibodies on western blots, high hemagglutination activity, and similar HA protein concentrations in the ELISA assays. We expect production of antigen specific antibodies against the four HA antigens by observing increased HI antibody titers after two vaccine doses.

MATERIALS AND METHODS

Mice vaccinations for this study were conducted at Nobel Life Sciences, Inc. in Sykesville, Maryland, USA. All other experiments were performed at Medigen, Inc. in Frederick, Maryland, USA.

Analyzing and Quantitating Hemagglutinin (HA) Composition in Virus-Like Particles (VLP's)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Filter sterilized VLP proteins were prepared in 2X Laemmli buffer and ran on 4-12% SDS-PAGE (Genscript, Piscataway, NJ). After rinsing in de-ionized water three times for 10 minutes each, gels were stained with GelCode Blue for 1 hour (Thermo, Waltham, MA). After de-staining for a minimum for 5 hours, images were captured by camera. Protein molecular weight marker See Blue Plus 2 (Thermo, Waltham, MA) was used to determine protein band sizes.

Western Blot

Filter sterilized VLP proteins were prepared in 2X Laemmli buffer and ran on 5-12% SDS-PAGE prior to transferring on a nitrocellulose membrane. After blocking for 1 hour, primary antibody (either anti-H5, anti-H7, anti-H9, or anti-H10) was added and incubated for 1 hour at room temperature. Anti-H5 (A/turkey/Germany/R2472/2014) (H5N8), Anti-H7 (A/Shanghai/1/2013) (H7N9), and Anti-H9 (A/Hong Kong/33982/2009) (H9N2) mouse monoclonal antibodies were purchased from Immune Technology (New York, NY). Anti-H10 (A/Jiangxi-Donghui/346/2013) (H10N8) polyclonal antibody was purchased from Sino Biological (Wayne, PA). Next, anti-mouse secondary antibody (KPL, Gaithersburg, MD) was added and incubated for an additional 1 hour. The membranes were

developed by adding phosphatase substrate (KPL, Gaithersburg, MD), and images were captured by camera.

Qubit Assay for Total Protein Quantitation

Qubit reagents and standards were prepared by adding 10 μL of each standard, 5 μL of a 500 μg/mL bovine serum albumin (BSA) control, and 5 μL of VLP to each Qubit assay tube. To the standards, 190 μL of Qubit reagent master mix was added, while 195 μL was added to the samples. After incubating in the dark for 15 minutes at room temperature, concentrations were read using Qubit 2.0 Fluorometer (Thermo, Waltham, MA).

Hemagglutination Assay

After labeling each column of a V-bottom 96 well plate, 50 μL of 1XPBS was added to each well. To the first column, 50 μL of VLP sample was added, and 2-fold serial dilutions were performed using a multichannel pipettor. The last column was left blank with only 1XPBS as a negative control. After removing excess bubbles using a clean pipette tip, 50 μL of 0.5% turkey red blood cells (RBC's) from Lampire Biological (Pipersville, PA) were added to all wells. Images were captured by camera at 30, 45, and 60 minutes using a lightbox.

Enzyme-linked immunosorbent assay (ELISA)

Standard HA proteins and VLP samples were diluted to a 20 μg/mL starting concentration and 2-fold serial dilutions were performed for 12 wells. HA standard proteins H5 (A/Vietnam/1203/2004), H7 (A/Anhui/1/2013), H9 (A/HongKong/33982/2009), and H10 (A/Jiangxi-Donghui/346/2013) were purified at Medigen, Inc. (Frederick, MD). Samples were denatured by incubating at 60^{o}C for 20 minutes and 100uL of each antigen was immediately added to microtiter plates (Thermo, Waltham, MA). Plates were covered

with adhesive plastic and incubated at 4ºC overnight. Next, plates were washed three times by filling wells with 150 μL PBS-T (0.05% Tween-20) for 3 minutes each. Wells were coated with 200 μL of blocking buffer (5% non-fat dry milk in PBS-T) for 2 hours at room temperature then washed twice with PBS-T for 2 minutes. To each well, 100 μL of 1:500 diluted primary antibody (anti-H5, H7, H9, or H10) was added and incubated for 2 hours at room temperature. Plates were washed three times with 150 μL of PBS-T for 2 minutes. Next, 100 μL of 1:1000 diluted anti-mouse secondary antibody was added in blocking buffer and incubated for 1 hour at room temperature. Plates were washed three times with PBS-T for 2 minutes and twice with 1XPBS for 1 minute. To each well, 100 μL of p-Nitrophenyl-phosphate (pNPP) substrate was added (Sigma, St. Louis, MO). Using a microplate reader, measurements were taken at 0, 30, 45, and 60 minutes at 405nm wavelength. Plates were stored at 4ºC and a final reading was taken at 22 hours. All assays were done in duplicate.

Vaccination of Mice with VLP's

Vaccination Regimen

Forty 8-week-old female BALB/c mice were received at Noble Life Sciences from Charles River Laboratories and divided into three groups of eight in addition to two control groups. Group 1 was injected with multi-subtype VLP (6g VLP). Group 2 was injected with a blend of four single HA VLP's with each VLP expressing one HA (H5, H7, H9, H10) subtype, and Group 3 was injected with a single H10 VLP. Serum samples were collected via retro-orbital sinus at Day 0 and 28. After serum samples were collected, mice were subcutaneously injected with 250 μL dose of designated vaccine. At Day 56, terminal serum samples were collected, and mice were euthanized. Samples were stored at -80ºC.

Hemagglutination Inhibition Assay (HI) to Determine Antibody Titer

Antigen Preparation

Single HA (H5, H7, H9, H10) VLP's expressing only one HA subtype on the surface of the VLP were used as antigens. A hemagglutination assay (HA) was first performed as described earlier to establish the antigen dilution in hemagglutination units (HAU). The HA titer was determined by the last well where complete hemagglutination occurred and designated as 1 HAU. Two well dilutions before this titer was considered 4 HAU. The volume of antigen needed was then divided by the titer at 4HAU. VLP antigen was diluted in cold sterile 1X PBS.

Serum Preparation

To reduce unspecific binding, serum samples were diluted at 1:3 ratio with Receptor Destroying Enzyme (Denka-Seiken, Tokyo, Japan) and incubated at 37^{o}C for 20 hours. Next, RDE was deactivated by incubating samples at 56^{o}C for 1 hour.

After RDE treatment, 200 μL of 1X PBS was added to establish a 1:8 serum starting dilution. Using labeled 96-well V-bottom plates, 25 μL of cold 1X PBS was added to each well and 50 μL of serum was added to the first column of every row. Two-fold serial dilutions were performed by transferring 25 μL volume. The negative control well contained 50 μL of 1X PBS. To the test wells, 25 μL of diluted antigen was added. After incubating on ice for 30 minutes, 50 μL of 1% turkey blood was added. Plates were incubated on ice and images were captured at 30, 45, and 60 minutes. The antibody titer in the serum was recorded as the last well with a visible red button.

Data Analysis

For ELISA data, a standard curve was prepared using the average absorbance readings of the HA protein standards and each HA protein concentration in the VLP was calculated using the regression equation on GraphPad Prism 9 software. Two-sample t-Tests were performed using Microsoft Excel for HI titer comparison.

RESULTS

Analysis and Quantitation of Hemagglutinin (HA) Composition in Virus-Like Particles (VLP's)

To confirm the presence of different HA proteins in the multi-subtype 6g VLP, SDS-PAGE and western blot analyses were performed using anti-H5, H7, H9, and H10 antibodies. As shown in Figure 3a, GelCode Blue stained gel showed the expected approximate sizes of HA and Bgag proteins as 64 kDa and 51 kDa, respectively. Individual HA proteins were detected by western blot using H5, H7, H9, and H10- specific antibodies as shown in Figure 3b.

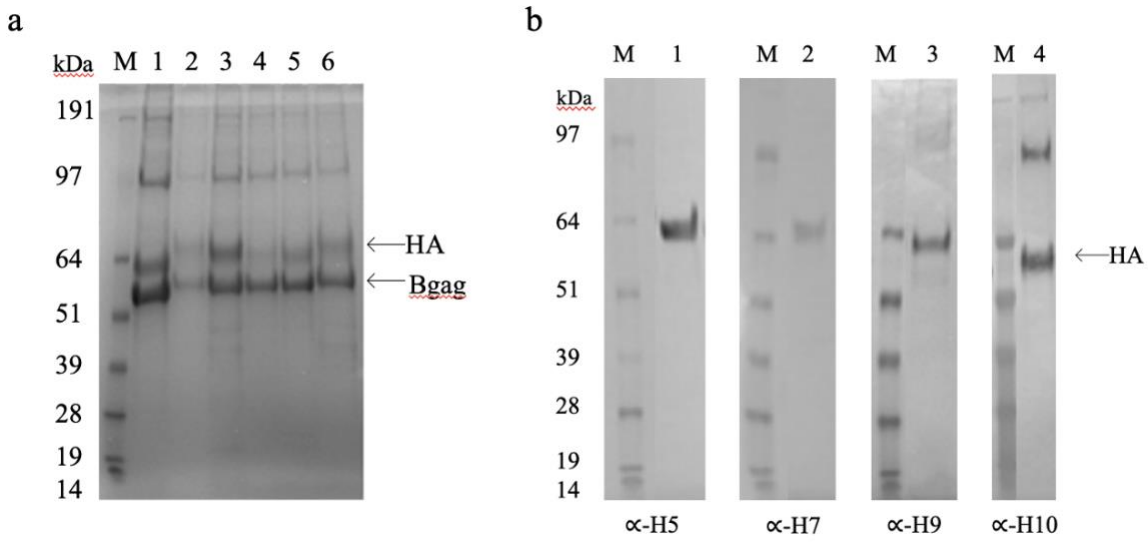

Figure 3. Detection of HA and Bgag proteins in 6g VLP. (a) GelCode Blue stained SDS-PAGE gel. Positions of HA and Bgag proteins are indicated by arrows. Marker (M) is See Blue Plus 2 Protein Standard. Lanes 1-6 indicate several VLP constructs; 1) H7(Hong Kong) VLP lot 2019.9.30, 2) H9(Hong Kong) VLP lot 2019.10.11, 3) H2(Canada) VLP lot 2019.09.05, 4) H5(Sichuan) VLP lot 2019.09.26, 5) 6g VLP lot 2019.09.19, and 6) NA-Less 6g VLP lot 2019.09.09. (b) Western blot analysis of 6g VLP using anti-H5, H7, H9, and H10 antibodies. Lane 1 is H5 protein in VLP detected by anti-H5 MAb, Lane 2 is H7 detected by anti-H7 MAb, Lane 3 is H9 detected by anti-H9 MAb, and Lane 4 is H10 detected by anti-H10 PAb. H9 and H10 Western blots prepared under non-reducing conditions (no 2-mercaptoethanol).

Hemagglutination assay and Qubit 2.0 assay

To confirm the hemagglutinin activity of the HA proteins in the VLP, an HA assay was performed at a 1:16 starting dilution. As shown in Figure 4, the HA titer was 1:2048. Additionally, the total VLP protein concentration was measured in duplicate by Qubit 2.0 Fluorometer. As shown in Table 1, the total protein concentration was 394 μg/mL while the BSA control concentration was 500 μg/mL.

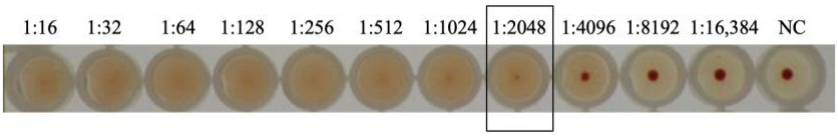

Figure 4. Hemagglutination assay of 6g VLP using 1% turkey RBC diluted in 1X PBS starting at a 1:16 dilution. The HA titer is indicated as 2048.

Table 1. Qubit 2.0 Fluorometer reading of total protein concentration in 6g VLP and BSA control samples. 6g VLP concentration was read in duplicate and a 500 μg/mL concentration of BSA protein was used as a control.

Qubit Sample	Concentration (ug/mL)	Average
New 6g Pand VLP Final 1	406	394 ug/mL
New 6g Pand VLP Final 2	382	
BSA 500ug/mL	500	

ELISA

To quantitate the HA concentrations in the 6g VLP, an indirect ELISA was performed by using anti-H5, H7, H9, and H10 antibodies along with purified single HA proteins as standards. GraphPad Prism 9 software was used for interpolating data by creating a hyperbolic curve of the standards as shown in Figure 5a. As shown in Figure 5b and Table 2, after an hour of pNPP substrate incubation, H5 protein concentration in the given 20 μg/mL sample of VLP was 0.248 μg/mL, H7 protein concentration was 0.171

μg/mL, H9 protein concentration was 23.454 μg/mL, and H10 protein concentration was 0.592 μg/mL.

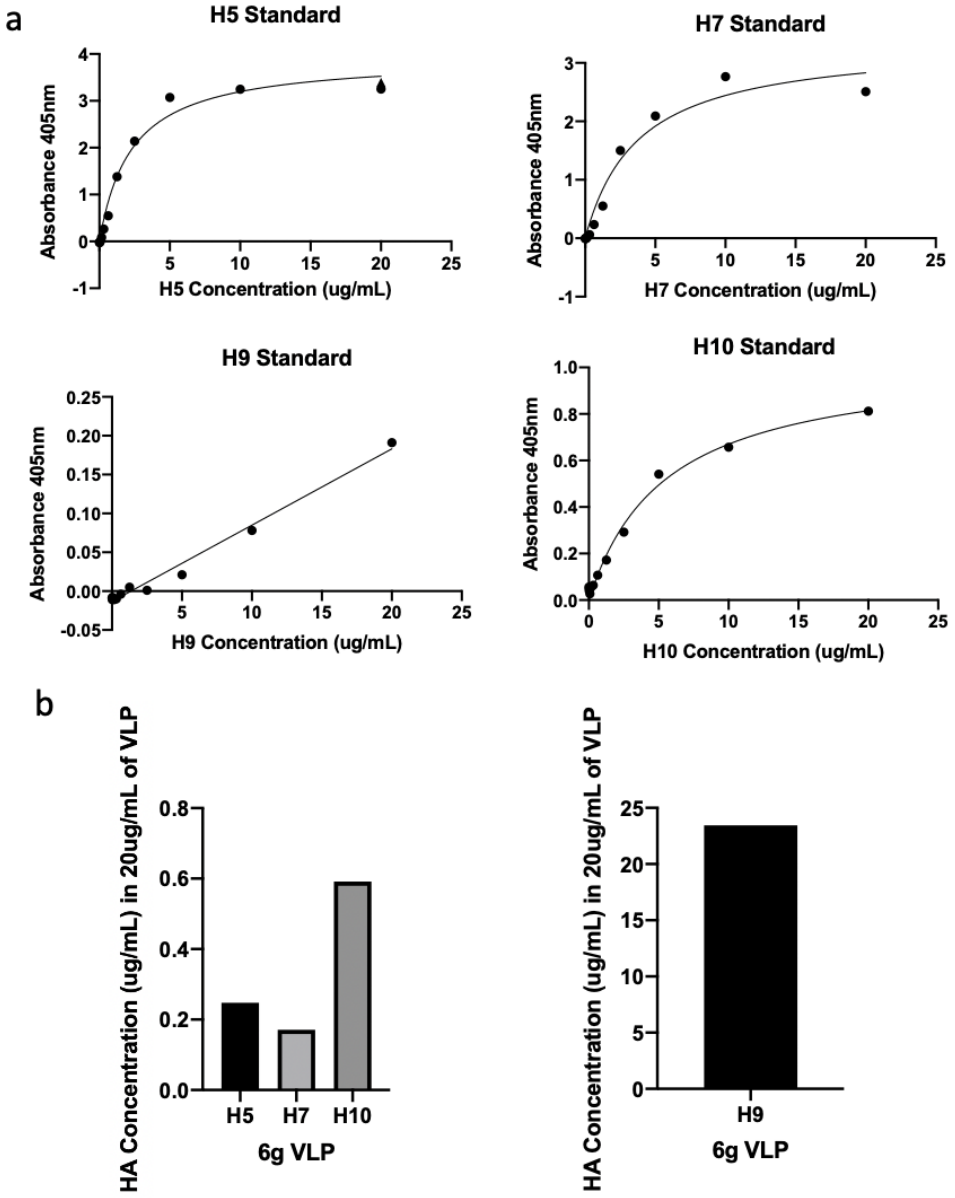

Figure 5. ELISA analysis of HA concentration (ug/mL) in a 20 μg/uL sample of 6g VLP using GraphPad Prism. (a) Standard curve of absorbance readings at 405nm after 1 hour for H5, H7, H9, and H10 protein standards. (b) Bar graph of H5, H7, H9, and H10 protein concentrations in 6g VLP after 1 hour incubation with pNPP substrate.

Table 2. Table summarizing HA concentration in 20 μg/mL of 6g VLP for ELISA data shown in Figure 5b.

	HA Concentration (ug/mL) in 20ug/mL of VLP			
	H5	H7	H9	H10
6gVLP	0.248	0.171	23.454	0.592

Vaccination of Mice with VLP's

Hemagglutination Inhibition (HI) Assay

To test the immunogenicity of the 6g VLP, female BALB/c mice were subcutaneously injected with either the 6g VLP, a blend of VLP's (H5(Si) VLP, H7(HK) VLP, H9(HK) VLP, and H10(JX) VLP), or the single H10 VLP (3gH10(JX)). The blended and single HA VLP's serve as comparative controls to determine if the 6gVLP is able to elicit a better immune response in mice. Serum samples were obtained at Days 0, 28, and 56 and stored at -80^{o}C. Sera were treated with RDE to reduce any unspecific binding. Single HA VLP's listed previously in the blended vaccination group were used as antigens to determine if the vaccinated mice produced HA-specific antibodies. As shown in Figure 6 and Table 3, Day 0 HI titers were at or less than 1:8 as expected for all groups except H9 VLP antigen.

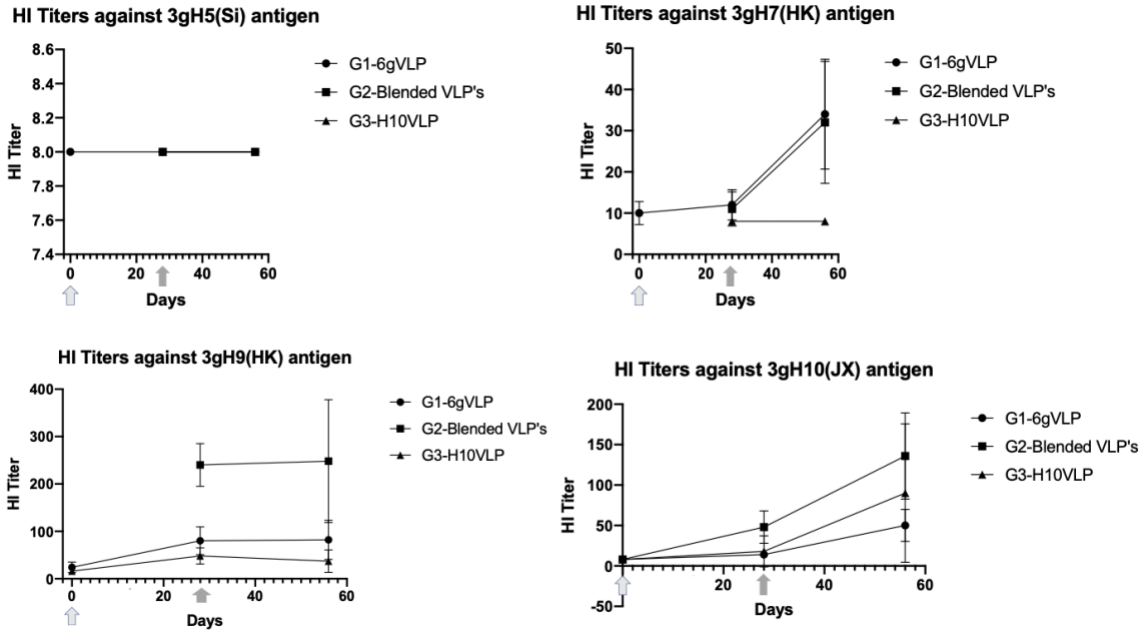

Figure 6. Hemagglutination inhibition assay for Day 0, 28, and 56 serum samples for mice subcutaneously injected with either 6g VLP, Blended VLP's, or H10VLP. HI titers against H5, H7, H9, and H10 VLP antigens were plotted using GraphPad Prism.

Table 3. Summary of Day 0, 28, and 56 mean HI titers against H5, H7, H9, and H10 VLP antigens for 6g VLP, Blended VLP's, and H10 VLP vaccination groups.

		Mean HI Titer		
Antigen	Group	Day 0	Day 28	Day 56
3gH5(Si)	1-6gVLP	8	8	8
	2-Blended VLP's	N/A	8	8
	3-H10VLP	N/A	8	8
3gH7(HK)	1-6gVLP	10	12	34
	2-Blended VLP's	N/A	11	32
	3-H10VLP	N/A	8	8
3gH9(HK)	1-6gVLP	24	80	82
	2-Blended VLP's	N/A	240	248
	3-H10VLP	16	28	37
3gH10(JX)	1-6gVLP	8	14	50
	2-Blended VLP's	8	48	136
	3-H10VLP	8	18	80

Table 4. t-Test: Two-Sample Assuming Unequal Variances between average HI titers against H7 antigen in 6g VLP and Blended VLP vaccinated mice groups.

t-Test: Two-Sample Assuming Unequal Variances between average HI titers against H7 antigen in 6gVLP and Blended VLP vaccinated mice groups.		
	Group 1	Group 2
Mean	34	32
Variance	178.285714	219.428571
Observations	8	8
Hypothesized Mean Difference	0	
df	14	
t Stat	0.28365431	
P(T<=t) one-tail	0.39041346	
t Critical one-tail	1.76131014	
P(T<=t) two-tail	0.78082692	
t Critical two-tail	2.14478669	

Table 5. t-Test: Two-Sample Assuming Unequal Variances between average HI titers against H9 antigen in 6g VLP and Blended VLP vaccinated mice groups.

t-Test: Two-Sample Assuming Unequal Variances between average HI titers against H9 antigen in 6gVLP and Blended VLP vaccinated mice groups.		
	Group 1	Group 2
Mean	82	248
Variance	1714.28571	16896
Observations	8	8
Hypothesized Mean Difference	0	
df	8	
t Stat	-3.4417281	
P(T<=t) one-tail	0.00440068	
t Critical one-tail	1.85954804	
P(T<=t) two-tail	0.00880137	
t Critical two-tail	2.30600414	

Table 6. t-Test: Two-Sample Assuming Unequal Variances between average HI titers against H10 antigen in 6g VLP and Blended VLP vaccinated mice groups.

t-Test: Two-Sample Assuming Unequal Variances between average HI titers against H10 antigen in 6gVLP and Blended VLP vaccinated mice groups.		
	Group 1	Group 2
Mean	50	136
Variance	397.7142857	2852.571429
Observations	8	8
Hypothesized Mean Difference	0	
df	9	
t Stat	-4.266607331	
P(T<=t) one-tail	0.00104536	
t Critical one-tail	1.833112933	
P(T<=t) two-tail	0.00209072	
t Critical two-tail	2.262157163	

None of the groups showed HI titer increase against H5(Si) VLP antigen. 6g VLP group showed increased HI titer against H7, H9, and H10 VLP antigens. Unexpectedly, H10 VLP group showed a mean HI titer of 37 against H9 antigen. Overall, Blended VLP group showed higher HI titers for all antigens compared to the 6g VLP and H10VLP groups. As summarized in Tables 5 and 6, Two-tail t-test analyses showed significantly higher HI titers for Blended VLP group compared to 6g VLP group against H10 and H9 antigens ($P<0.05$). However, as shown in Table 4, both groups did not show significantly different HI titers against H7 antigen ($P=0.780$).

DISCUSSION

Influenza remains a serious global public health threat. Avian influenza viruses of H5, H7, H9, and H10 are of particular concern because of their pandemic potential. A vaccine capable of protecting against multiple potentially pandemic influenza viruses would be of great benefit for public health worldwide. VLP's present alternatives for producing broadly protective vaccines against potential pandemic avian influenza subtypes.

In this study, we confirmed the presence of H5, H7, H9, and H10 hemagglutinin glycoproteins within the 6 gene VLP construct by performing SDS-PAGE, western blot, HA, and ELISA assays. SDS-PAGE staining showed Bgag protein at approximately 51 kDa and HA protein at 64 kDa. Western blots using anti-H5, H7, H9, and H10 monoclonal antibodies confirmed the presence of all four HA subtypes in the 6g VLP suggesting the potential of VLP's to induce immune response to all four subtypes. The protein sizes along with positive western blot bands are consistent with results from the multi-subtype VLP generated previously (Tretyakova et al. 2016). Although the 1:2048 titer is lower compared to 1:8192 titer from the previous VLP, the functional ability of the 6g VLP to agglutinate to turkey RBCs was still confirmed (Tretyakova et al. 2016).

ELISA assay also confirmed and quantitated HA concentrations in the 6g VLP. While H5, H7, and H9 concentrations ranged between 0.2 to 0.5 μg/mL, H9 concentration was greater than 20 μg/mL in the given 20 μg/mL of VLP. Although the same GraphPad Prism algorithm was applied to all data sets, H9 data yielded higher concentrations. In contrast, semi-quantitative western blot analysis of previous multi-subtype VLP yielded roughly 25 percent of each HA (Tretyakova et al. 2016).

One explanation for these results lies in the sensitivity of the antibody-antigen interaction. HA protein standards (purified using a histidine trap column at Medigen, Inc.,) must be highly purified. Impurities could interfere with the Qubit 2.0 Fluorometer protein concentration readings. Also, the HA protein standards and the monoclonal antibodies used must match precisely. The available monoclonal antibodies used for antigen detection were not exactly matching the strain of HA used in the VLP and the HA standard. For example, anti-H5 (A/turkey/Germany/R2472/2014) (H5N8) antibody was used against H5 (A/Vietnam/1203/2004) protein standard. There are resource limitations for obtaining antibodies against the latest specific avian HA.

The ELISA assay developed at Medigen, Inc. specifically for VLP quantitation could still need fine-tuning. For example, VLP and standard HA proteins were denatured at 60^{o}C for 20 minutes prior to adding on microtiter plates. Denaturation temperature and time may vary between different HA, which would need to be adjusted.

Pipette instrument and human error may have contributed to dilution error. In addition, the quality of the HA standards could have played a role. HA proteins were stored at -20^{o}C and may have undergone multiple freeze-thaws, which could denature the proteins. Antibodies were stored at 4^{o}C and the shelf life may vary with each antibody impacting the sensitivity and cross reactivity. To avoid these errors in the future, HA standards should be prepared individually without serial dilutions to reduce concentration error. HA standards should be analyzed on SDS-PAGE and western blot to confirm protein stability and reactivity to HA-specific antibody.

After confirming the presence of all HA subtypes in the 6g VLP, an immunization study was performed to determine if the VLP could generate an immune response in mice.

In addition to the 6g VLP, a blended VLP consisting of single HA VLP's (H5,H7,H9,H10) and a single H10 VLP were also used for testing. Since the 6g VLP contained all four HA subtypes, it was expected that the VLP would elicit HA-specific antibodies against all four subtypes.

HI results show production of HA-specific antibodies against all but H5 antigen for 6g VLP-vaccinated mice. This suggests the broadly protective capabilities of 6g VLP as potential vaccine against multiple pandemic subtypes. Blended VLP and H10 VLP groups showed low HI antibody titer against H5(Sichuan) VLP antigen, as well. This data is similar to prior findings in a study using trivalent VLP construct containing H5, H7, and H9 where low HI titers against H5 were observed in the initial first vaccine dose (Tretyakova et al. 2013). Low immunogenicity of H5 has been previously observed (Treanor et al. 2006, Wong et al. 2017).

The blended vaccination group showed highest HI titers against all HA antigens with the exception of H5 antigen. One possible reason could be because a single HA VLP has more surface area or antibody binding opportunity compared to a VLP with multiple different HA's. There could also be competition or interference between different HA's on the 6g VLP. Additionally, for 6g VLP, the different HA's may not be expressed in equal abundance. For example, this 6g VLP construct may contain predominantly H9 antigen and limited quantities of other subtypes. The 6g VLP was designed to express each HA in equal abundance on its surface with each HA gene having its own upstream polyhedrin promoter. However, there could be HA protein degradation along with other factors in VLP assembly and secretion in insect cells, which is beyond the scope of this study.

Although blended vaccines show greater efficacy in this study, generating a multi-subtype VLP would be more cost effective than a blend of multiple single HA VLP's. Four separate manufacturing processes would be needed for creating a blended vaccine while the multi-subtype VLP would be generated in single process. The multi-subtype VLP dosage could be increased to match efficacy of the blended vaccine, which would still be more cost effective. To further compare of the two vaccine groups, a challenge study with live influenza virus should be performed on vaccinated groups to look for any cross-reactive protection against different influenza subtypes. Further research would be required to optimize H5 immunogenicity with possible adjuvants.

Here we showed that a multi-subtype VLP was able to elicit HI antibody titers against H7, H9, and H10 subtypes, with the exception of low HI titer against H5, from avian influenza viruses. There were limitations to available antigens and antibodies. Nevertheless, we showed that analyzing and quantitating HA content in VLP's was an achievable goal with several variables in each assay needing fine adjustments. These findings further support the promising opportunity of multi-subtype VLP's as potential candidates for broadly protective pandemic influenza vaccines.

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