EVALUATION OF SINDBIS-M2E VIRUS VECTOR AS A UNIVERSAL INFLUENZA A VACCINE

A Thesis

by

CHRISTINE NGUYEN VUONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Biomedical Sciences

Evaluation of Sindbis-M2e Virus Vector as a Universal Influenza A Vaccine

Copyright 2012 Christine Nguyen Vuong

EVALUATION OF SINDBIS-M2E VIRUS VECTOR AS A UNIVERSAL INFLUENZA A VACCINE

A Thesis

by

CHRISTINE NGUYEN VUONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee, Blanca M. Lupiani
Committee Members, Sanjay M. Reddy
Michael Criscitiello
Head of Department, Linda Logan

August 2012

Major Subject: Biomedical Sciences

ABSTRACT

Evaluation of Sindbis-M2e Virus Vector as a Universal Influenza A Vaccine.

(August 2012)

Christine Nguyen Vuong,

B.S., Texas A&M University

Chair of Advisory Committee: Dr. Blanca M. Lupiani

Although avian influenza virus (AIV) infections in domestic poultry are uncommon, transmission of avian influenza from wild waterfowl reservoirs does occur. Depopulation of the infected flock is the typical response to AIV outbreaks in domestic chicken production, causing a loss in profits and accumulation of unexpected expenses. Because it is impossible to know which of many virus subtypes will cause an outbreak, it is not feasible for the U.S. to stockpile vaccines against all possible avian influenza threats. Currently, the U.S. does not routinely vaccinate chickens against influenza due to the inability to differentiate infected from vaccinated animals (DIVA), which would place limitations on its trade markets. A Sindbis virus vector expressing the PR8 influenza strain's M2e peptide was developed as a potential universal DIVA vaccine. M2e is a conserved peptide amongst influenza A viruses; M2e-specific antibodies induce antibody-dependent cytotoxicity or phagocytosis of infected cells, reducing production and shedding of AIV during infection. In this study, chickens were vaccinated at one-month-of-age with parental (E2S1) or recombinant Sindbis viruses

expressing the PR8 M2e peptide (E2S1-M2e) by subcutaneous or intranasal routes at high (10^6 pfu) or low (10^3 pfu) dosages. Chickens were boosted at 2-weeks post-initial vaccination using the same virus, route, and dosage, then challenged with low pathogenic H5N3 AIV at 0.2 mL of 10^6/mL EID_{50} 2-weeks post-boost. Serum samples were collected at 1-week and 2-weeks post-vaccination, 2-weeks post-boost, and 2-weeks post-challenge and screened for PR8 M2e-specific IgY antibody production by ELISA. Both high and low dose subcutaneously, as well as high dose intranasally vaccinated E2S1-M2e groups produced significantly higher levels of PR8 M2e-specific IgY antibodies as early as 1-week post-vaccination, while the uninoculated control and E2S1 groups remained negative for all pre-challenge time points. M2e-specific IgY antibodies capable of binding the challenge H5N3 M2e peptide were detected in groups with existing vaccine-induced M2e-specific antibodies pre-challenge, suggesting antibody M2e cross-reactivity. After challenge, all groups developed M2e-specific IgY antibodies and high HI titers, verifying successful AIV infection during challenge and production of hemagglutinin-specific antibodies. Viral shedding titers 4-days post-challenge were used to measure vaccine efficacy and were similar amongst all groups. Microneutralization assay results confirmed that post-boost serum samples, containing only M2e-specific antibodies, were unable to neutralize AIV *in vitro*. Although the E2S1-M2e vaccine was capable of producing high levels of M2e-specific IgY antibodies when inoculated subcutaneously, these antibodies were not able to reduce viral shedding and therefore did not protect chickens from AIV.

ACKNOWLEDGEMENTS

First, I would like to thank my committee members Drs. Blanca Lupiani, Sanjay Reddy, and Michael Criscitiello. Thank you all for your patience and unwavering support in both calm and stressful times. I would not have survived the graduate student experience without your guidance and encouragement. Thanks for the opportunities to broaden my experience outside of research and providing interesting hallway conversation. To Dr. Hans Heidner for designing the E2S1-M2e vaccine vector and allowing me to perform the evaluation trial in chickens, thank you for sharing your work. This study could not happen without you.

Thanks to Dr. Pamela J. Ferro for her help in all things big and small, for she is truly a person of unfailing kindness and a fountain of knowledge. Thank you for always having the time to guide me through a problem, lend an ear when I am low, and take the time to support me through stressful endeavors. You will never know how much I value your friendship. To my lab mates Drs. Owais Khan and Aijun Sun, thank you for your kindness, for your instruction in the laboratory, and hours spent teaching me proper poultry husbandry in the middle of a Texas summer.

To my parents, thank you for your tolerance and endurance through my many years in school and for your patience in the years to come. To my sister Ann, an inadvertent passenger on the journey I have taken, thank you for surviving this experience with me.

NOMENCLATURE

Ad	Adenovirus
AIV	Avian Influenza Virus
AGID	Agarose Gel Immunodiffusion
cRNA	Positive Strand RNA, Complementary RNA
DIVA	Differentiating Infected and Vaccinated Animals
E1	Envelope Protein 1
E2	Envelope Protein 2
E2S1	Parental Sindbis Virus
E2S1-M2e	Influenza M2e-Expressing Sindbis Virus
E3	Envelope Protein 3
EID_{50}	50% Embryo Infective Dose
ELISA	Enzyme-Linked Immunosorbent Assay
HA	Hemagglutinin
HEPA	High-Efficiency Particulate Arresting
HI	Hemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
HRP	Horseradish Peroxidase
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL-2	Interleukin 2

IL-4	Interleukin 4
LPAI	Low Pathogenic Avian Influenza
M1	Matrix Protein
M2	Membrane Bound Ion Channel-like Protein
MDCK	Madin Darby Canine Kidney Cells
NA	Neuraminidase
NDV	Newcastle's Disease Virus
NEP	Nuclear Export Protein
NAHLN	National Animal Health Laboratory Networks
NP	Nucleocapsid Protein
NPIP	National Poultry Improvement Plan
NS1	Non-Structural Protein 1
NVSL	National Veterinary Services Laboratory
OD	Optical Density
PA	Polymerase Acidic
PB1	Polymerase Basic 1
PB2	Polymerase Basic 2
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05% Tween 20
Pfu	Plaque Forming Unit
PR8	A/Puerto Rico/8/1934/H1N1
RBC	Red Blood Cells

RNA	Ribonucleic Acid
RNP	Ribonucleoproteins
rRT-PCR	Real-time Reverse Transcriptase Polymerase Chain Reaction
SPF	Specific Pathogen Free
$TCID_{50}$	50% Tissue Culture Infective Dose
Th2	Type II Helper T-Lymphocyte
TPB	Tryptose Phosphate Broth
USDA	United States Department of Agriculture
UTSA	University of Texas at San Antonio
vRNA	Viral RNA

TABLE OF CONTENTS

Page

ABSTRACT iii

ACKNOWLEDGEMENTS v

NOMENCLATURE vi

TABLE OF CONTENTS ix

LIST OF FIGURES x

1. INTRODUCTION 1

   1.1. Avian influenza virus 1
   1.2. Genome and replication 3
   1.3. Vaccines 10
   1.4. M2 protein and M2e peptide 19
   1.5. Sindbis virus vectors 23
   1.6. E2S1-M2e vaccine vector 25
   1.7. Hypothesis 26
   1.8. Objective 26
   1.9. Significance 26

2. E2S1-M2e VACCINE EFFICACY IN CHICKENS 28

   2.1. Introduction 28
   2.2. Materials and methods 30
   2.3. Results 36
   2.4. Discussion 46

3. SUMMARY 52

REFERENCES 53

LIST OF FIGURES

FIGURE Page

1 Cryo-electron tomography of parental E2S1 Sindbis virus and recombinant E2S1-M2e vaccine virus 25

2 Pre-challenge PR8 M2e-specific IgY ELISA results 37

3 Pre-challenge and post-challenge PR8 M2e-specfic IgA ELISA results ... 38

4 PR8 M2e-specific IgY levels pre- and post-challenge with low pathogenic H5N3 AIV 40

5 Pre- and post-challenge PR8 M2e-specific IgY levels in comparison to viral shedding of challenge virus 41

6 M2e-specific IgY cross reactivity to H5N3 M2e peptide pre-challenge.... 42

7 M2e-specific IgY cross reactivity to H5N3 M2e peptide post-challenge .. 43

8 Hemagglutination inhibition titers of post-challenge serum 45

1. INTRODUCTION

1.1. Avian influenza virus

Avian influenza virus (AIV) was originally described in northern Italy as "fowl plague" by Edward Perroncito during an outbreak in poultry, confusing the disease with an acute septicemic form of avian cholera (2, 15, 84, 100). Based on clinical and pathological properties, the disease was shown to be different and renamed *typhus exudatious gallinarium* in 1880 (100). By 1901, the causative agent was shown to be an ultra-filterable agent, a virus (22). The association between the characterized fowl plague and other low pathogenic influenza A viruses isolated from birds and mammals was not demonstrated until 1955 by Schäfer, when he was able to serologically detect type A influenza viral ribonucleoproteins in fowl plague samples (93).

Wild aquatic birds are considered the natural reservoirs for type A influenza viruses and all type A influenza subtypes have been identified in these species (97, 123). Typically, AIV infections in wild waterfowl are asymptomatic, but outbreaks due to highly pathogenic avian influenza viruses have been described (55). AIV can infect other avian hosts, such as domestic chickens and turkeys, which can result in large economic losses in production and trade (2, 36). Outbreaks of multiple AIV subtypes have affected chickens throughout the years, resulting in depopulation of flocks and loss of revenue (43, 45). Outbreaks of AIV in turkeys have primarily been of the H3, H7, and H9 subtypes (45). Multiple influenza outbreaks have occurred amongst turkeys in the United States due to turkey susceptibility to both avian and swine origin influenza

___

This thesis follows the style of Avian Diseases.

viruses (130). Swine-origin triple reassortant influenza viruses are a common problem in turkeys (77, 109). Pigs can host human, avian, and swine influenza, and are capable of generating triple reassortant viruses (59, 127). Influenza outbreaks in turkeys primarily occur in the Minnesota, North Carolina, and Utah due to their close proximity to swine operations (45, 54, 71). This has been a tremendous problem for the turkey industry, as they attempt to control the spread of infection while maintaining bird numbers and breeder flocks.

The presence of both low pathogenic avian influenza and highly pathogenic avian influenza in poultry flocks has been detected multiple times worldwide in domestic poultry farms since 1929 (28, 44, 54, 56). Highly pathogenic strains cause clinical, systemic disease capable of 100% mortality in poultry, while low pathogenic strains cause mild to asymptomatic respiratory and enteric tract infections as well as a decline in egg production (80). Low pathogenic strains are more commonly detected during AIV outbreaks in domestic chickens and turkeys, however several outbreaks of highly pathogenic strains have occurred (44, 106, 107). As depopulation of the exposed flock is often the response to highly pathogenic avian influenza (H5 and H7 subtypes) outbreaks, significant economic losses can occur. This may mean the loss of egg production from an entire flock of layers, loss of pounds of broiler meat, loss of breeder flocks, and loss of exportation markets for live birds or by-products (43, 91). Vaccination is rarely used as a response to chicken AIV outbreaks in the United States as it places limitations on trade and exportation. Importing countries are unwilling to accept vaccinated poultry because these birds cannot be differentiated from infected

birds (43, 91), but the vaccination strategy has been an increasingly recommended method to prevent and control AIV infections in domestic poultry (17).

1.2. Genome and replication

Avian influenza is a type A influenza virus of the *Orthomyxoviridae* virus family, which is characterized by its segmented, negative-sense single-stranded ribonucleic acid (RNA) genome. The *Orthomyxoviridae* family consists of the *Thogotovirus*, *Isavirus*, and *Influenzavirus A*, *B*, and *C* genera (80). Type A influenza viruses are capable of infecting avian and mammalian hosts, while types B and C are typically limited to mammalian hosts (127). Influenza virus particles are enveloped and pleomorphic, varying in shape from spherical to filamentous (69). Their genome consists of eight negative-polarity RNA segments encoding for 10-12 viral proteins depending on the strain. The three largest segments, segments 1-3, encode for the RNA-dependent RNA polymerase complex: polymerase basic 1 and 2 (PB1 and PB2) and polymerase acidic (PA), which are required for viral genome transcription and replication. The hemagglutinin (HA) and neuraminidase (NA) are important antigenic determinants and are encoded by segments 4 and 6; these two proteins project on the surface of the virus particle and are critical for attachment (HA) and release (NA) of the virion. The fifth segment encodes the viral nucleoprotein (74), which interacts with the viral RNA and the polymerase complex to form the eight ribonucleoproteins (RNP). Segment 7 codes for two proteins: the matrix protein (M1) which coats the inside of the viral envelope and the transmembrane ion channel-like protein (M2) (80). The eighth segment encodes two proteins: the non-structural protein 1 (NS1) and nuclear export protein (NEP), which are

important for decreasing host gene expression and exportation of the viral RNPs from the nucleus, respectively.

Infection begins when the influenza HA protein attaches to its host cell receptor, sialic acid (42, 96). Upon HA-sialic acid binding, the influenza virus enters the cell via receptor-mediated endocytosis (58). Once in the endosome, the M2 ion channel protein allows the influx of H^+ ions into the virion. This influx of H^+ ions decreases the pH, disrupting protein-protein interactions within the virion and leads to the release of viral RNP from the M1 protein. The low pH of the endosomal environment also activates HA protein's fusion properties, leading to fusion of the viral membrane with the endosomal membrane, allowing for the release of RNP into the host cell cytoplasm. Viral RNPs are then transported to the nucleus where viral mRNA synthesis and genome replication are catalyzed by the viral RNA-dependent RNA polymerase complex, consisting of the viral PB1, PB2, and PA proteins. (39). The viral genome replication occurs by synthesis of positive-sense full length copies of viral RNA called complementary RNA (cRNA) and subsequent copying of cRNA into negative-sense viral RNA (vRNA). The synthesis of viral mRNA requires host cellular RNA polymerase II because a 5' capped primer is required. The 5' capped primer is obtained from newly synthesized host cell mRNA by the "cap snatching" mechanisms carried out by the viral polymerase complex (80). Translation of the M2, HA, and NA envelope proteins from mRNAs, occur in the rough endoplasmic reticulum of the host cell. These viral envelope proteins produced then undergo post-translational modification, pass through the Golgi for additional modifications, and are transported to the apical cell surface of polarized epithelial cells.

The other viral mRNAs (PB1, PB2, PA, NP, NS1, and M1) are translated on free ribosomes and brought back into the nucleus to associate with viral RNAs to form RNPs, which are then exported out of the nucleus and through the cytoplasm to associate with the envelope proteins. Once all viral components are present at the apical surface of the infected host cell, the viral components assemble into viral particles and release from the host cell by budding out of the plasma membrane. Newly formed viral particles are released from the cell surface by NA cleaving of sialic acid, therefore not allowing the HA of newly formed influenza virions to become bound and stuck to the surface of the host cell from which the virus is being released (80).

1.2.1. AIV classification

Influenza viruses are classified based on their HA and NA surface glycoproteins. There currently are 16 HA subtypes (H1-16) and 9 NA subtypes (N1-9), which permits a possible 144 different influenza combinations based on HA and NA alone (80). Avian influenza viruses are further classified by virulence by denoting strains as low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) (15). LPAI is associated with low morbidity and typically do not result in mortality, while HPAI strains are defined by their high morbidity and/or mortality in chickens (127). The H5 and H7 AIVs are the only known subtypes capable of becoming HPAI, but not all H5 and H7 subtypes are HPAI (2). Pathogenicity testing of AIV can be based either by virus challenge in chickens or by sequence analysis (3). During AIV pathogenicity index testing in chickens, if 6-8 out of 8 AIV inoculated chickens die, the virus is considered HPAI, but if no birds die during challenge the virus is considered LPAI (3).

If 1-5 birds die, the influenza virus is considered moderately pathogenic (3). Genetic-based classification of pathogenicity is based on the influenza hemagglutinin (HA) cleavage site sequence. The AIV HA cleavage site is typically cleaved by the exogenous protease trypsin, limiting infection to the respiratory and gastrointestinal tracts because trypsin in only present at those sites (127). HPAI contains a polybasic amino acid sequence at its HA cleavage site, allowing the AIV to be cleaved by furin-like proteases (121). Furin is ubiquitous throughout the body, allowing for systemic infection of AIV containing a polybasic HA cleavage site (127).

1.2.2. Mechanism of mutations of the AIV genome

Genetic mutations in the influenza genome can allow the virus to evade the immune system of the host despite prior exposure. AIV genome changes occur either by genetic drift or genetic shift (123). Genetic drift is the accumulation of point mutations during normal viral replication due to the viral RNA polymerase's lack of proofreading mechanism, and is the most common method of change. These point mutations typically result in influenza viruses associated with epidemics, such as seasonal influenza infections in humans, as protection developed against the past year's influenza strain may not provide protection against the currently circulating strains. Genetic shift occurs when more than one influenza virus replicates in the same host and in the same cell, allowing genome segments to switch between the strains during viral assembly (123). This type of genomic mutation is quite rare and is typically how pandemics strains are generated, such as the 1957 H2N2 Asian flu pandemic (87, 129), the 1968 H3N2 Hong Kong pandemic (87), and the 2009 H1N1 pandemic (99).

AIV infections in the United States' domestic chickens are typically not a problem (123). Typical commercial poultry operations in the United States do not expose their domestic flocks to wild bird reservoirs or practice mixed-species production on its facilities, reducing the risk of AIV exposure (44). Upon transmission into chickens, mutations in the AIV genome can allow adaptation of the virus to the chicken host (11, 12). Avian influenza outbreaks do occur on rare occasions in the United States, and are typically of low pathogenic strains (44, 54, 56, 126). Upon survival and passage in chicken hosts, AIV genome mutations can produce a HPAI (11, 12, 66). The standard method of response to an AIV outbreak in chickens is culling of the infected flock (107).

1.2.3. AIV screening in the United States

Routine serological screening is the current surveillance method used by the poultry industry (118). As recommended by the United States Department of Agriculture's (118) National Poultry Improvement Plan (74), flocks are screened for AIV at 90-day intervals through serological or viral isolation methods by the National Animal Health Laboratory Network (NAHLN) (3). NAHLN laboratories conduct early avian influenza surveillance in the United States and have the capacity to screen a large number of samples during an outbreak (3). The agarose gel immunodiffusion assay (AGID) is a highly specific test that detects antibodies against the type specific influenza antigens NP and M1 and is considered the gold standard serological screening method for AIV (74, 118), but results can sometimes be difficult to read and subjective. The hemagglutination inhibition (HI) assay, which detects HA-specific antibodies, is another test that can be used to screen for exposure to avian influenza by detecting HA-specific

antibodies (118). The HI assay can also be used for quick HA subtyping to detect antibodies produced against the potential HPAI subtypes, H5 and H7 (127). These serological assays are used for routine surveillance because they are both cost effective and can be easily used to screen multiple samples. If H5 or H7 HPAI is suspected in a flock, RNA isolation of the influenza virus genome and real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) targeting the influenza matrix gene is the preferred method of diagnosis, followed by pathogenicity testing (3). Real-time RT-PCR amplification is a more sensitive assay method because it is able to detect actual influenza gene segments. Because this assay method detects the virus, viral isolation and subsequent rRT-PCR testing is the preferred method during or when an AIV infection is suspected. In contrast, AGID is used for routine surveillance because this assay detects the production of antibodies against AIV, which occurs after infection. Positive rRT-PCR results for influenza in state laboratories must be further tested by National Veterinary Services Laboratory (NVSL) in Ames, Iowa to confirm results (3, 74). Pathogenicity testing consists of 0.2 mL intravenous injection of 4-8 week old naïve birds with 1/10 dilution of the influenza virus sample in bacteria-free allantoic fluid and observing mortality rates (3). Should there be mortality in 6-8 out of 8 birds within 10 days after inoculation, the virus is considered highly pathogenic. If there is no mortality, the influenza virus is considered lowly pathogenic. If the influenza induced mortalities between 1-5 out of 8 birds is considered moderately pathogenic (3). In contrast to LPAI, moderately pathogenic AIV can result in mortality rates ranging from

5-97%, with the highest mortality in young or stressed birds, as well as cause lesions in the respiratory tract and various gastrointestinal organs (19, 137).

Confirmatory and pathogenicity testing is particularly important when an AIV outbreak occurs, so a suitable response to the outbreak can be quickly implemented. An outbreak of H7N2 AIV occurred in 2003 on a commercial layer farm in Connecticut; this virus was classified as LPAI (91). An approximate 1.3 million layers were in production and cost of depopulation was estimated to exceed $30 million by the Connecticut Department of Agriculture (91). To reduce losses, Connecticut opted to vaccinate its flocks with inactivated H7N2 influenza vaccine; vaccinating both the already infected hens as well as naïve replacement pullets (91). Due to the 15 months length of each layer production cycle, and the time necessary for the farm to maintain a zero infection rate, it would take several years for this farm to obtain an AIV-free status. In February 2004, an AIV outbreak occurred in a commercial broiler flock with an estimated 6,600 birds in Gonzales County, Texas (83). Birds were tested positive for AIV based on initial diagnosis via AGID assay and the virus was identified as an H5N2 subtype; the H5N2 strain was classified as HPAI based on sequence analysis (83). Depopulation of the flock was performed to prevent potential spread of this H5N2 virus, but after completion of pathogenicity index testing, no mortality was observed in the test birds (83). Depopulation of the infected flock after the initial HPAI classification was necessary to prevent spread of a potentially high pathogenic avian influenza infection, as an outbreak response cannot wait for further pathogenicity test results.

1.3. Vaccines

Transmission of AIV into domestic poultry is economically and politically detrimental to the industry; almost 9 billion pounds of broiler meat and 91 billion eggs were processed in the United States in 2010 alone (119). Currently, the United States poultry industry does not vaccinate against avian influenza due to the low probability of infection and the expense of vaccination. Currently used AIV-screening assays are unable to differentiate between infected and vaccinated birds, which would limit the United States' ability to export its poultry if current vaccines are used (44, 78, 104). Other countries without major export markets and endemic AIV infections in their domestic poultry have chosen to vaccinate their flocks with inactivated influenza virus vaccines (25, 85). The vaccination strategy has been used in the United States during AIV outbreaks in chickens on rare occasions, instead of the usual depopulation strategy (43). AIV inactivated vaccines were used during the 1995 H7N3 AIV outbreak in Utah (43), and again during the 2002 H7N2 AIV outbreak in layer chicken breeder flocks in Connecticut (91). In both of these cases, vaccination was able to dramatically reduce the expected economic loss by over 50% based in comparison to previous outbreak losses and the industry was not forced to cull the entire breeder chicken flock (43). It is not a routine practice to vaccinate turkeys against AIV, but vaccination was used when the swine-origin H3 triple reassortant influenza strain infected a turkey flock during an outbreak in Minnesota (27). The use of vaccination decreased mortality amongst the birds, but did not become a routine practice in the United States overall (67). Because of their close proximity to swine operations, repeated outbreaks of AIV have occurred in

Minnesota and North Carolina turkey operations (45, 130). Since avian influenza vaccination requirements for turkey production are not as strict as vaccination requirements in chickens, some commercial producers in the state have opted to vaccinate their turkey flocks on a routine basis.

1.3.1. Inactivated and alive-attenuated vaccines

Inactivated/killed vaccines have had moderate efficacy in poultry (79, 112) and have been used with great success to prevent many avian diseases affecting the industry. The advantages of an inactivated AIV vaccine include uniform levels of immunity, no spread of the virus, and little to no adverse reactions (67). Despite these advantages, the current inactivated AIV vaccines induce poor protection in the presence of maternal antibodies which would be problematic should breeder hens be vaccinated with inactivated AIV vaccines or were naturally infected before lay in countries with endemic AIV (32). In one particular study, chicks carrying anti-H5 AIV maternal antibodies were vaccinated with inactivated H5 AIV vaccine and produced lower antibody levels compared to chicks without maternal antibodies; these chicks also excreted higher levels of HPAI H5N1 virus during challenge (32). Inoculation with inactivated AIV vaccine at one-day-of-age also induced poor protection in chicks with and without maternal antibodies based on clinical signs, HI titers, and challenge viral shedding, suggesting vaccination with inactivated AIV vaccines at one-day-of-age is ineffective (32).

Alternatively, live-attenuated vaccine viruses are able to replicate in the host and thus induce strong immune responses (21, 108), but have some drawbacks. Live-attenuated vaccines are not approved for use in chickens, because vaccinated chickens

are still able to shed the vaccine virus which may regain virulence after passages within the chickens (78), but experimental studies have been performed in poultry (21). Four-week-old specific pathogen free (SPF) chickens were infected with a laboratory-passage attenuated H7N3 AIV to study viral shedding titers and antibody production via HI assays compared to the unattenuated H7N3 AIV strain (21). Reduced viral shedding was observed 3 and 6 days post-inoculation in birds vaccinated with the attenuated AIV, this strain was also able to induce similar antibody production levels compared to the unattenuated strain (21). Despite the ability to induce high antibody levels, live-attenuated vaccines have never been seriously considered for commercial poultry use as these vaccines are associated with decreased production due to respiratory disease, have the potential to revert back to virulence, and can spread from flock to flock (7, 63).

1.3.2. AIV and DIVA vaccines

Avian influenza vaccines currently used are based on inactivated influenza virus. A problem with this vaccination strategy is that vaccinated birds cannot be differentiated from infected birds with currently used avian influenza detection assays, which would limit a country's ability to trade its poultry with other countries. The standard AGID serological screening assay relies on NP and M1-specific antibody detection (16). The HI assay screens for HA-specific antibodies. Antibodies against avian influenza HA, NP, and M1 proteins would be present in birds vaccinated in the current whole-virus inactivated vaccines as well as birds naturally infected with AIV, and therefore both vaccinated and infected birds would be positive for AIV using the AGID and HI assays. Due to these limitations, countries importing live poultry and poultry products are

reluctant to accept poultry immunized with the current inactivated vaccine since these birds cannot be differentiated from infected birds (70). The development of DIVA (differentiation of infected and vaccinated animals) strategies in relation to influenza vaccines are currently underway and would help resolve some of the issues associated with vaccination and screening assays. Upon successful development of an avian influenza DIVA vaccine, AIV endemic countries could vaccinate with fewer concerns about trade implications. Some DIVA strategies involve the use of 1) vaccination with homologous HA proteins to the circulating AIV strain, but with a heterologous NA protein (5), 2) serological response measurement to NS1 protein (53), or 3) vaccination with vectored vaccines (13, 37).

Heterologous NA vaccination in birds would allow differentiation of infected birds by detecting NA-specific antibody production; vaccinated birds should produce antibodies against the recombinant vaccine NA protein, but not the NA of the circulating AIV strain. Naturally infected birds would not develop antibodies against the recombinant NA using for vaccination. The use of heterologous NA vaccination against AIV was tested and used in Italy as a response to the H7N1 avian influenza epidemic of 1999-2000 (18). This H7N3 vaccine tested for field use was capable of providing protection upon challenge with an H7N1 HPAI based on reduced viral shedding as well as reduced viremia (presence of infectious AIV in muscles) in chickens (20). N1-specific antibodies were detected by indirect immunofluorescent antibody testing using recombinant N1 expressing baculovirus (20). Although vaccination with a homologous HA protein and heterologous NA protein may decrease viral shedding and viremia

during infection, this vaccination method is not ideal for use in the poultry industry. Application of a heterologous NA style vaccine would be effective provided only one other NA subtype is circulating at a time in a given area; this is rarely the case in real world situations. If the current circulating/outbreak AIV strain contains the same NA as the vaccine, vaccinated and infected birds could not be differentiated, defeating the purpose of this DIVA strategy (20).

Several approaches have been used to develop NS1 deficient vaccines in hopes of developing a successful DIVA vaccine. The NS1 protein, an influenza virus virulence factor, interferes with type I interferon responses in the host, decreasing the effectiveness of the innate immune response (52, 95). The NS1 strategy uses absence or presence of NS1-specific antibodies to differentiate between vaccinated and infected birds, respectively. One study measured sera NS1-specific antibody levels via enzyme-linked immunosorbent assays (ELISA) obtained from AIV infected birds, inactivated commercial vaccinated birds, and purified inactivated AIV vaccinated birds. Birds vaccinated with purified inactivated AIV vaccine did not develop antibodies to NS1 while AIV infected birds possessed high NS1-specific antibodies (116). Chickens vaccinated with unpurified inactivated commercial AIV vaccines possessed low, but detectable, levels of NS1-specific antibodies. Upon challenge infection with a low pathogenic AIV, all chickens were able to produce NS1-specific antibodies, but these antibody levels rapidly decreased within 5 weeks (4). The rapid decrease of NS1-specific antibodies induced by natural AIV infection rendered this DIVA strategy ineffective since naturally infected chickens cannot be differentiated from vaccinated

birds 5 weeks past the initial time of infection (4, 101). Commercial scale avian influenza vaccine manufacturing for poultry includes non-structural proteins, such as the NS1 protein, rendering clear differentiation between vaccinated and infected birds impossible. With this in mind, a vaccination study using a vaccine virus containing a mutant NS1, a truncated NS1 without the immunogenic carboxyl end, to differentiate between infected and vaccinated birds was conducted (10). H5N3 AIV infected birds produced NS1-specific antibodies, while birds vaccinated with the H5N3 NS1 mutant vaccine virus did not produce antibodies against NS1, as the immunogenic region was removed in the NS1mutant. This mutant NS1 vaccine was able to reduce viral shedding, based on viral RNA levels, but the NS1 protein regained virulence upon back passage in chickens (10).

Additional DIVA vaccines for AIV have been developed using fowl poxvirus (14, 26, 86), Newcastle's disease virus (88, 94), turkey herpesvirus (64, 89), and adenovirus (68, 114) as vaccine vectors for use in poultry. AIV vaccination using a viral vector delivery system allows for dual vaccination, a bivalent vaccine, against both AIV and the viral delivery system; this allows birds to develop immunity against the actual virus vector used and AIV (120). Vaccines are already developed against fowlpox, Newcastle's disease virus, and Marek's disease, but bivalent vaccines capable of providing protection against two diseases at once would reduce the amount of vaccines administered and reduce vaccination expenses. Virus vector vaccines have the potential for DIVA capabilities. Vaccinated birds would develop antibodies to only the AIV protein included in the bivalent vaccine, but not develop antibodies to any of the other

avian influenza virus proteins, such as the NP or M1 proteins. Because the AGID assay used to screen for avian influenza infection targets NP and M1-specific antibodies, vaccinated birds would be negative for AIV infection during screening.

TROVAC-H5, a live recombinant fowlpox vaccine expressing the H5 hemagglutinin protein, has full registration in Mexico, El Salvador, and Guatemala and is licensed for emergency use in the United States (14). When vaccinated into 1-day-old SPF chicks, the TROVAC-H5 vaccine was able to protect against morbidity, mortality, and reduce viral shedding upon challenge with H5N2 HPAI 3-weeks after vaccination (103). Protection was conferred up to 20 weeks after vaccination, showing TROVAC-H5 provided long-lasting immunity. All vaccinated birds were negative upon AGID testing for AIV, showing its potential use as a DIVA vaccine (103). Another trial using fowlpox expressing the H5 protein from H5N1 HPAI was able to induce high HI titers after vaccination in 4-week-old SPF chickens, and provide protection against homologous challenge 3-weeks post-vaccination based on reduced viral shedding and survival of all chickens (86). Fowlpox-based vector vaccines against AIV are ineffective when birds have been previously vaccinated or exposed to fowlpox (102), but are still effective in the presence of maternally-derived antibodies (14).

Attempts have been made to develop bivalent Newcastle's disease virus vector systems for H5 AIV vaccination (37, 75, 120) and H7 AIV vaccination (105). The NDV-H5 vector vaccine was approved for use in China and developed using reverse genetics to insert the AIV H5 gene between the NDV matrix and phosphoprotein genes of the currently used Newcastle's vaccine strain, LaSota (37). Both the NDV-H5 and

NDV-H7 vaccines induced protection against both NDV and AIV based on reduced mortality upon lethal challenge against NDV and HPAI, and the induction of high HI levels compared to unvaccinated chickens (37, 105, 120). The NDV-H5 live-attenuated vaccine was able to induce antibody production and provide protection upon challenge with homologous and heterologous H5 AIV subtypes (37, 75). Although the eye-drop inoculation method was the only method used during experimental testing, these vaccines may allow for spray inoculation to vaccinate large chicken flocks at once, eliminating the need for individual inoculation of birds by trained personnel. A disadvantage to the NDV vector method would be that if the birds have pre-existing immunity to the vector delivery system, as the vaccine vector would be cleared before successfully inducing an immune response to the AIV protein (94). This is also true when recently hatched chicks contain high levels of maternally-derived antibodies, because the vaccine vector again will be cleared from the chicks' system before inducing a protective immune response (32). Upon vaccination with NDV-H5, SPF chickens were protected against both NDV and H6N2 LPAI upon challenge based on reduced viral shedding, but turkeys exhibiting anti-NDV maternal antibodies at vaccination time had only marginally reduced viral shedding titers (94). Therefore the NDV vector vaccination strategy cannot be used for birds previously vaccinated against NDV, birds already naturally infected, or birds with high levels of maternally-derived antibodies.

Turkey herpesvirus (HVT) has been used to vaccinate chickens against Marek's disease for over 50 years. Recently, an HVT vector system with an HA insert was developed by Ceva for use in avian influenza endemic countries. Ceva's Vectormune

HVT-AIV vaccine has passed requirements for USDA registration as a genetically-engineered vaccine (23). The HVT-AIV vaccine has been shown to be effective in the presence of maternally-derived antibodies (31) and provides protection against both Marek's disease and AIV based on reduction of clinical signs and reduced viral shedding (in both level of viral excretion and number of shedding birds) (31). Because maternally-derived antibodies do not decrease vaccine effectiveness, the HVT-AIV vaccine can be inoculated at one-day-of-age as well as *in ovo* (24). Herpesvirus infections are life-long and because HVT-AIV is administered as a live vaccine, the vaccine-induced immunity is life-long. Upon homologous challenge with a H7N1 HPAI, HVT-H7 vaccinated turkeys had reduced viral excretion and reduced mortality (64). Five out of 7 vaccinated birds survived the challenge with H7N1 HPAI, while none of the unvaccinated turkeys survived the challenge (64). When comparing the HVT-H5 vaccine with the Ceva-Mexico produced inactivated H5N2 vaccine, HVT-H5 vaccinated turkeys developed heterologous protection between two antigenically different HPAI H5N1 strains circulating in Egypt from 2007 and 2008 (89). HVT-H5 vaccinated birds had increased HI titers, no mortality, and reduced viral shedding upon challenge (89). Vaccination with inactivated H5N2 vaccine produced by Ceva-Mexico showed only a delay in bird death after challenge with the 2008 Egypt HPAI H5N1 when compared to unvaccinated turkeys. Heterologous protection against the 2007 Egypt H5N1 could not be tested in the inactivated H5N2 vaccinated birds due to bird mortality (89).

Replication-defective recombinant adenovirus (Ad) has also been successfully used as a delivery vector for AIV vaccination (68, 114). Ocular vaccination of commercial layer chickens with adenovirus encoding the H5N9 HA (Ad-H5) at 5 days of age and boosted at 15 days produced high levels of H5-specific antibodies at 32 days of age based on HI assay (114). Birds singly vaccinated, without booster, maintained antibody levels similar to unvaccinated control birds. Upon challenge with H5N2 HPAI at 42 days of age, no mortality was observed in Ad-H5 boosted birds as well as significantly reduced challenge viral shedding based on quantitative RT-PCR (114). In another study using the Ad-H5 vaccine, SPF chickens were vaccinated *in ovo* at 18 days of embryonation and challenged with H5N2 HPAI at 42 days of age (68). Reduced viral shedding was observed via quantitative RT-PCR in vaccinated birds as well as increased survival. Antibody levels were measured via HI assay, showing persistent antibody levels up to 18-weeks-of-age. Chickens without detectable levels of antibody were also protected from challenge based on survival and reduced viral shedding (68), supporting the recent finding that adenovirus vectors used for AIV vaccines elicit effector and memory CD8+ cytotoxic T lymphocytes (98). Chickens vaccinated *in ovo* in the presence of maternally-derived antibodies did not seroconvert, indicating interference of vaccine efficacy in the presence of high levels of maternally-derived antibodies (68).

1.4. M2 protein and M2e peptide

1.4.1. M2 protein characterization

M2 protein is a conserved protein among influenza A viruses, which homotetramerizes to form the transmembrane ion channel necessary for successful viral

uncoating and subsequent replication in the host cell (50). The influenza M2 ion channel protein allows an influx of H^+ ions into the virion, which breaks protein-protein interactions, and allows the viral RNPs to be released into the host cell cytoplasm. M2 protein is expressed at low levels on the influenza virion surface and at higher levels on infected host cells (60, 132). Due to M2's low expression levels on the virus surface, natural infection with AIV does not induce a strong M2-specific immune response from the host. Typical immune responses against AIV target the HA protein, resulting in the production of neutralizing HA-specific antibodies allowing the host to clear the infection, but mutations in the HA protein frequently occur via antigenic drift which can render prior subtype-specific immunity ineffective. Because M2's coding sequence overlaps with the influenza A M1 coding sequence, which is highly conserved, mutations rarely occur in the M2 sequence (60). Based on M2's highly conserved nature amongst influenza A viruses, antibodies against M2 should provide cross-protection against different influenza A virus subtypes. The M2e peptide is the N-terminus extracellular epitope of the M2 protein and may be a suitable target for universal avian influenza vaccines (60, 132). Although the mechanism by which M2e-specific antibodies act is unclear, mouse studies have shown M2e-vaccines induce type II T helper (Th2) cells, activating antibody production, which goes on to opsonize infected cells to induce antibody-dependent cytotoxicity or phagocytosis *in vivo*, thus reducing viral production and protecting against full avian influenza infection (34, 51). M2e-specific antibodies do not prevent AIV infection from occurring, but are able to provide protection based on reduced viral shedding during infection (34, 35, 51).

1.4.2. M2e-based vaccines

M2e-peptide based vaccines have been designed for many host systems, with studies predominantly performed in mice. Because M2e peptide possesses low immunogenicity (9, 51, 125), the peptide must to be inoculated in conjugation with highly immunogenic proteins or delivery systems, or be provided in multiple copies in order to induce a significant immune response (134). Most studies have shown M2e-specific antibodies opsonize infected cells to induce phagocytosis (34, 48, 131). One study states antibody-dependent cytotoxicity occurs via natural killers cells (51), while another study refers to the exclusive use of alveolar macrophages for phagocytosis of the infected host cell (34). Whichever mechanism it may be, transfer of M2e-vaccinated mouse serum containing M2e-specific antibodies reduced viral shedding, and thus provided protection against influenza challenge in mice (35, 115). Recent studies have shown a large involvement from Th2 cells based on the increased levels of interleukin-4 (IL-4) cytokine secretion in M2e-vaccinated mice (1, 135). Upon depletion of Th2 cells from vaccinated mice, survival rates dropped almost 50% (1). These results help support previous antibody response mechanisms proposed, confirming Th2 cells are necessary to activate antibody responses in the host system. Because M2e-specific antibodies act by reducing viral production, not neutralization of the virus, M2e-based vaccine efficacy is measured by reduction in viral shedding during infection.

Previous influenza M2e-based vaccination studies have had mixed results; M2e fused to hepatitis B core proteins has been very successful in mice (76, 133), but did not provide protection in swine (47). Two phase-I human trials have been conducted to test

single-epitope vaccine immunogenicity by vaccinating patients and measuring M2e-specific antibody production levels at various time points after vaccination. M2e-specific antibody production was the only indicator for the human phase-I trials, as controlled challenge studies cannot be conducted on human participants. Both human trials, using hepatitis B core-M2e fusion from Sanofi Pasteur Biologics (73) or flagellin-M2e fusion from VaxInnate Corp. (72, 117), were successful in these immunogenicity studies. Several trials have been conducted using multiple epitope vaccines, vaccines containing multiple influenza protein epitopes, in order to stimulate cytotoxic T-cell involvement. Dynavax used a combination influenza NP and M2e vaccine in order to induce production of both M2e-specific and NP-specific antibodies, which have been shown to induce cytotoxic T cell involvement. This vaccine safely stimulated production of antibodies during phase-I human trial. (33). Biondvax developed an HA, NP, and M1 epitope vaccine shown to safely induce both humoral and cell-mediated immunity during phase I and II human trials based on production of antibodies and activation of cellular immunity by interferon gamma (IFN-γ) and interleukin 2 (IL-2) secreting T-lymphocytes (8).

AIV vaccination trials in chickens have been quite successful. *Salmonella* expressing an M2e and immune-enhancing CD154 peptide fusion (61) was able to induce a strong M2e-specific immune response in chickens. The *Salmonella*-M2e conjugate vaccine induced M2e-specifc antibody production and provided full protection upon challenge with low pathogenic H7N2 AIV based on reduced clinical signs and shedding, but not against highly pathogenic AIV. Another trial vaccinated chickens with

a M2e-*E. coli* maltose-binding protein fusion vaccine (134). The M2e-fused to *E. coli* maltose-binding protein vaccine was able to reduce mortality rates in chickens upon lethal challenge with highly pathogenic H5N1 AIV, providing partial protection against AIV. Both M2e vaccine studies in chickens provided partial to full protection against AIV after conjugation with more immunogenic partners, based on antibody production levels, reduced morbidity/mortality during challenge and reduced viral shedding. These studies are particularly important because most M2e-based vaccination trials and efficacy data are primarily performed in mice, not chickens, which have differing immune system mechanisms.

1.5. Sindbis virus vectors

Sindbis virus is an *Alphavirus* of the *Togaviridae* family, characterized by its unsegmented positive-sense RNA genome (40). The nsP1-nsP4 nonstructural genes are encoded at the 5' end of the viral genomic RNA while the capsid and envelope structural proteins are encoded from subgenomic messenger RNA, transcribed from the center of the genome and extended to the 3' end. The surface of the Sindbis virion is covered by propeller-like projections made of the envelope 1 (E1) and envelope 2 (E2) glycoproteins. The E1 and E2 proteins form an E1-E2 heterodimer, which further trimerizes to form each surface projection. With 80 projections on the Sindbis viral surface, a total of 240 E1 and 240 E2 proteins are expressed. Both the Sindbis E1 and E2 glycoproteins are highly immunogenic; E1-specific antibodies tend to cross-react with other alphaviruses, while E2-specific antibodies are virus specific (i.e. Sindbis virus) (40). As an arbovirus, Sindbis' transmission cycle alternates between an

arthropod vector, in this case the mosquito, and a vertebrate host. Sindbis is naturally inoculated intravenously and replicates in avian species as its vertebrate host. This infection is asymptomatic, causing no clinical morbidity or mortality in the birds and would therefore not cause reduced production after vaccination with this vector (40). Sindbis virus has also been established as a safe and convenient expression system, capable of expressing large numbers of foreign viral proteins fused to its E2 glycoprotein, and is not considered a significant human pathogen (40).

1.5.1. Sindbis as a vaccine vector

Many vaccines have used Sindbis as a delivery system with effective results (6). Sindbis has proven to be a successful vaccine vector against measles by expressing measles virus hemagglutinin and fusion proteins, which provided full protection upon challenge in rhesus macaques (81). Another vaccine using Sindbis vector expressing glycoprotein G of rabies virus has also been successfully shown to provide protection against Rabies infections in dogs (41, 92). One study used Sindbis to express anthrax's protective antigen as a potential anthrax vaccine (111). Although this Sindbis-anthrax protective antigen vaccine vector was able to induce antibody production, the antibodies produced did not provide protection upon challenge with anthrax in mice. Despite this inability to provide protection, the anthrax vaccine was able to enhance the protective effects of ciprofloxacin, an antibiotic commonly used to treat anthrax. Because this virus naturally infects avian species (40), Sindbis virus provides an excellent platform for avian influenza vaccination in chickens and would allow differentiation between vaccinated and infected birds.

1.6. E2S1-M2e vaccine vector

1.6.1. E2S1-M2e design and production

A Sindbis virus expressing the M2e peptide was designed and produced at the University of Texas at San Antonio (UTSA) in Dr. Hans Heidner's laboratory. The A/Puerto Rico/8/1934/H1N1 (PR8) M2e peptide gene (SLLTEVETPIRNEWGCRCNGSSD) was inserted into the Sindbis genome upstream from the E2 glycoprotein gene. The envelope 3 (E3) gene was removed so the M2e peptide became the N-terminus of the E2 protein. This insertion allowed the PR8 M2e peptide to be expressed as a fusion peptide on the surface of each Sindbis E2 envelope glycoprotein. This created a 240 M2e dense Sindbis virus (Fig. 1) without affecting viral replication (46).

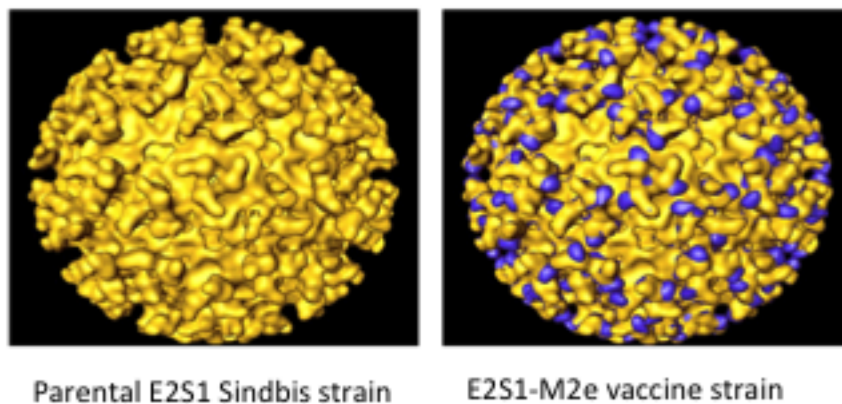

Fig. 1. Cryo-electron tomography of parental E2S1 Sindbis virus and recombinant E2S1-M2e vaccine virus. M2e peptides are depicted in blue and fused to the Sindbis E2 surface glycoprotein.

The parental Sindbis virus delivery system used was the E2S1 virus strain; this Sindbis virus strain contains genetic alterations made on the E2 glycoprotein gene to

allow for expression of fusion peptides (110). The recombinant virus expressing the influenza M2e peptide was designated the E2S1-M2e vaccine virus. The E2S1-M2e virus is expected to easily infect and replicate in birds because avian species are the natural host for Sindbis virus infections. The dense M2e expression on the recombinant E2S1-M2e virus should overcome the influenza M2e peptide's natural low immunogenicity and induce a greater immune response in the host.

1.7. Hypothesis

Based on Sindbis virus' ability to cause asymptomatic infection in avian species and the recombinant E2S1-M2e virus expresses a high density of M2e peptides, we hypothesized that vaccination of chickens with E2S1-M2e virus will induce the production of M2e-specific antibodies and provide protection against AIV upon challenge with low pathogenic H5N3 AIV.

1.8. Objective

The specific objective of this study was to evaluate the recombinant E2S1-M2e virus as a potential AIV vaccine in chickens, with the long-term objective of developing a universal AIV type A vaccine based on the E2S1-M2e system.

1.9. Significance

Introduction of AIV into a poultry flock would have significant economic impact on the industry by reducing production rates or requiring depopulation of the flock to eliminate spread of the disease. Currently, the U.S. does not routinely vaccinate its chickens against influenza, primarily due to the low probability of AIV transmission into chickens makes vaccination not cost effective and the negative trade implications

because current inactivated AIV vaccines do not allow differentiation between infected and vaccinated animals. The use of the E2S1-M2e vaccine vector offers the capability to differentiate between infected and vaccinated birds while providing universal protection against AIV, a major advantage. A universal avian influenza DIVA vaccine would allow the United States to vaccinate its flocks without negatively effecting exportation. This vaccine could also be stockpiled for emergency use; because of E2S1-M2e's universal capabilities it can be of immediate use during poultry outbreaks, eliminating the time required for new vaccine reformulation, production, and distribution.

2. E2S1-M2e VACCINE EFFICACY IN CHICKENS

2.1. Introduction

Avian influenza is a respiratory and enteric disease caused by type A influenza viruses of the *Orthomyxoviridae* virus family (127). Type A influenza viruses are classified based on the two major surface proteins: hemagglutinin (HA) and neuramidase (NA); there are 16 HA subtypes and 9 NA subtypes. Wild waterfowl are the natural reservoirs for avian influenza viruses (AIV), but these viruses can infect other non-natural avian hosts such as chickens, turkeys, or quail (2). The introduction of AIV into a poultry flock would have significant economic impact on the industry by reducing production rates or requiring depopulation of the flock to eliminate spread of the disease, therefore causing loss of profits and incurring extra expenses during an outbreak (15).

The United States does not vaccinate its poultry flocks against avian influenza. Current inactivated AIV vaccines do not allow differentiation between infected and vaccinated birds by the presently used AIV-screening assays. Other countries will not import United States poultry if the vaccinated birds cannot be distinguished from infected birds. The low probability of AIV transmission into domestic poultry makes vaccination against AIV cost ineffective as well. Existing AIV vaccines provide only subtype-specific protection (14, 37), requiring continuous vaccine reformulation and production to remain current with the circulating influenza subtype. Stockpiles of H5 and H7 AIV subtype vaccines are retained in the United States because these subtypes are capable of becoming highly pathogenic, but vaccine stocks of the other 14 HA subtypes are not reserved (107). Should an avian influenza outbreak occur from these

other 14 HA subtypes, it would take months to reformulate and distribute a new vaccine. The development of a universal stock vaccine providing protection against multiple AIV strains, capable of differentiation between infected and vaccinated animals (DIVA), would eliminate the need and cost associated with vaccine reformulation, production, and distribution.

The influenza M2 protein is a highly conserved protein necessary for successful viral uncoating and replication of influenza A viruses (50, 127). This protein is expressed on avian influenza virions and on the surface of AIV infected cells (60). Based on previous studies, the conserved influenza M2e peptide, the M2 protein surface epitope, would provide an excellent target for vaccination and cross-protection between type A influenza subtypes (61). Because of M2e's low immunogenicity, M2e requires conjugation with a highly immunogenic protein or delivery system, or vaccination with high copy numbers. The use of a Sindbis virus as a vaccine vector to express the influenza M2e peptide would be ideal for use in chickens as this virus naturally replicates in avian species causing asymptomatic infections (40), and would allow for DIVA strategies. The vector vaccine developed for use in this study expresses 240 copies of influenza M2e peptide on the surface of a Sindbis virus (E2S1-M2e). The multiple copies of M2e would induce the development of universal type A influenza immunity, while the use of a Sindbis vector permits the use of DIVA strategies to differentiate vaccinated and infected chickens.

The objectives of this study were to determine immunogenicity of the Sindbis-M2e vector vaccine (E2S1-M2e) in chickens at different inoculation routes and dosages

and to determine vaccine efficacy based on reduced viral shedding upon challenge with low pathogenic H5N3 AIV.

2.2. Materials and methods

2.2.1. Generation of recombinant E2S1-M2e vaccine vector

The parental Sindbis virus designated E2S1 (46) and the E2S1-M2e virus were designed and developed by Dr. Heidner of UTSA. The PR8 M2e peptide sequence (SLLTEVETPIRNEWGCRCNGSSD) was inserted upstream of the Sindbis E2 gene with the E3 gene removed, allowing influenza M2e to be expressed as a fusion peptide on the N-terminus of the Sindbis E2 gylcoprotein, resulting in the E2S1-M2e virus. The fusion of M2e to the Sindbis E2 protein did not cause loss of viral infectivity (57) and created a virus expressing 240 copies of M2e on its surface.

2.2.2. Vaccination and challenge

Specific pathogen free eggs (Charles River Laboratories International, MA, USA) were incubated, hatched in SPF conditions within the laboratory, and moved to high-efficiency particulate arresting (HEPA) filtered isolation units for the duration of the study. Chickens were divided in eight different groups with 14 birds/group and vaccinated at 4-weeks-of-age. E2S1 and E2S1-M2e viruses were administered by the intranasal or subcutaneous routes, 50 µL and 200 µL respectively, at both high dose (10^6 pfu (plaque forming units)) or low dose (10^3 pfu) per chicken. Parental E2S1 virus was used during vaccination as a control to ensure the parental virus did not produce adverse effects in the hosts and to determine background for detection of M2e-specific antibodies. The ninth uninoculated group served as unvaccinated control. Chickens

were boosted two weeks post vaccination using the same dosage and route. Subcutaneous and intranasal routes of administration were examined as Sindbis viruses infect intravenously by transmission from mosquitos while influenza naturally replicates in the respiratory tract (29, 30, 49, 133). Immunoglobulin A (124) antibodies are produced in mucosal surfaces of the respiratory and gastrointestinal tracts while immunoglobulin Y (IgY) antibodies is secreted into the blood stream and therefore present in vasculated tissue, such as muscle. Influenza infections naturally reside in the respiratory and gastrointestinal tracts; therefore, protection against AIV requires high IgA antibody levels. All groups were challenged with 0.2 mL of 10^6/mL EID_{50} (50% embryo infective dose) low pathogenic A/Chicken/TX/02 H5N3 AIV two weeks post-boost by nasal, ocular, and intra-cloana routes.

2.2.3. Sample collection

Blood samples were collected from all chickens at one and two-weeks post-vaccination and two-weeks post-boost (one day before virus challenge). Blood (1 mL) was collected from the brachial vein and stored at 4°C overnight to allow the serum to separate from the red blood cells. Serum was collected and stored at -20°C until tested. Two-weeks post-boost, five birds from each group (4 birds in the control group) were euthanized by carbon dioxide asphyxiation and their tracheas collected into 2.5 mL phosphate buffered saline (PBS) to determine the presence of M2e-specific IgA antibodies. PBS containing tracheas were stored at 4°C, then vortexed to allow IgA into solution and the tracheas then removed from the vials within 5 hours of collection. Tubes were then centrifuged and the top 2 mL collected and stored at -20°C until tested.

Concentration of some tracheal wash samples was attempted using centrifugal microconcentrators (Sartorius Stedim Biotech, Aubagne Cedex, France) following manufacturer's instructions. Tracheal swabs were collected four days after challenge using sterile Dacron swabs (Fisher Scientific, Houston TX, USA) in 2.5 mL tryptose phosphate buffer (TPB; Becton Dickinson NJ, USA) supplemented with antibiotics/antimycotics [penicillin G ($1x10^4$ U/mL), streptomycin ($1x10^4$ μg/mL), and amphotericin B (25 μg/mL) (Invitrogen, Grand Island NY, USA)]. Samples were stored at -80°C until processed for RNA isolation. Blood samples (1 mL) were collected two-weeks post-challenge from the brachial vein and process as indicated above.

2.2.4. M2e-specific IgY and IgA ELISA

Serum samples were diluted to 1:50 with PBS and screened for M2e-specific IgY antibody levels by enzyme-linked immunosorbent assay (ELISA). Briefly, Immulon 1B medium-binding polystyrene plates (Thermo, TX, USA) were coated with 50 μL total volume of 2 μg/mL M2e peptide (Genscript, Piscataway NJ, USA) in carbonate buffer, pH 9.6, overnight at room temperature. The following morning, plates were blocked with 250 μL of 3% bovine serum albumin in PBS for 1 hour. Plates were then washed three times with 0.05% Tween 20 in PBS (PBST) using an automated plate washer (BioTek ELx50 Microplate Strip Washer, Fisher Scientific) before applying 50 μL of 1:50 diluted serum sample per well and incubating for 1 hour at room temperature. Positive control wells used mouse M2e-specific monoclonal antibody 14C2 (Santa Cruz Biotechnology, Santa Cruz CA, USA) against the N-terminus of the M2 protein of the A/Wisconsin/1933/H1N1 and was diluted to 1:500 in PBS and used as the primary

antibody. Negative control wells were treated with PBS to check for nonspecific binding of secondary antibodies. Plates were then washed three times with PBST and 50 μL of 1:10,000 secondary antibody was added to each well followed by one hour incubation at room temperature. Secondary antibody for chicken serum samples was horseradish peroxidase (HRP) conjugated goat anti-chicken IgY or IgA (Bethyl Laboratories, Montgomery TX, USA) and for mouse HRP-conjugated goat anti-mouse Ig H+L (Santa Cruz Biotechnologies, Santa Cruz CA, USA). After incubation with secondary antibody, plates were washed 5 times with PBST, and 50 μL of SureBlue TMB substrate (KPL, Gaithersburg MD, USA) was applied for 20 minutes at room temperature and optical density values read at 630 nm. Reactions were then stopped with 1N HCl and OD (optical density) values read again at 450 nm. ELISA was also performed on post-boost and post-challenge serum samples using plates coated with H5N3 specific M2e peptide (SLLTEVETPTRNGWECKCNDSSD) and following the same procedure. ELISA to determine presence of M2e-specific IgA in trachea washes followed the same procedure as IgY, but the sample remained undiluted. All serum samples were tested in triplicate.

2.2.5. rRT-PCR for viral titer

Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) was used to calculate viral titers based on the abundance of AIV matrix RNA in chicken tracheal swabs collected 4-days post-challenge. A standard curve was generated using RNA extracted from serial ten-fold dilutions of A/turkey/WI/68 H5N9 AIV virus stock of known titer, and used to calculate virus titers by extrapolation, as previously described

(62). RNA was extracted from tracheal swab samples using the MagMax™-96 Viral RNA Isolation Kit (Ambion, Austin TX, USA) following the manufacturer's protocol, and an automated extraction processor (Kingfisher 24, ThermoForma, Inc). A 1-step rRT-PCR, which detects the AIV matrix gene RNA, was performed using AgPath-ID™ AIV-M Reagent Kit (Ambion, TX) as per manufacturer's instructions.

2.2.6. Hemmagglutinin inhibition assays

Hemagglutination inhibition (HI) assays were performed to determine post-challenge antibody levels against challenge virus. Serum samples collected post-challenge were diluted with PBS by two-fold serial dilution up to 1:256. Twenty-five µL of each serum dilution was incubated for 30 minutes with 25 µL of H5N9 AIV containing 8 hemagglutinating units. After 30 minutes incubation at room temperature, 50 µL of 1% chicken red blood cells (RBCs) was added to each well and HI results read 45 minutes later. Virus with only RBCs and virus with negative serum and RBCs were used as negative controls. A chicken anti-H5 virus serum available in our laboratory was used as positive control.

2.2.7. Microneutralization assays

To test for M2e-specific neutralizing antibodies, microneutralization assays were performed on samples with high M2e-specific IgY OD values. H5N9 viral titer was determined by inoculating confluent monolayers of Madin Darby canine kidney (MDCK) cells with ten fold dilutions (8 replicates per dilution) of H5N9 virus stock followed by incubation at 37°C in a 5% CO_2 atmosphere for 4 days. Inoculated cells were observed daily for presence of cytopathic effect and virus titer calculated using the

method of Reed and Muench (90) and expressed as $TCID_{50}$ (50% tissue culture infective dose) units.

For the microneutralization assay, serum samples were first incubated with receptor-destroying enzyme (Hardy Diagnostics, CA, USA) at 37°C overnight at a 1:3 ratio respectively to eliminate possible non-specific inhibitors of HA activity in serum. The reaction was stopped by 45 minute incubation at 58°C and further diluted to 1:10 with PBS containing antibiotics/antimycotics. The 1:10 serum was further diluted (two-fold dilution up to 1:256) and 50 μL of each dilution incubated with 50 μL containing 100 $TCID_{50}$ of H5N9 AIV at 37°C for 30 min. The serum-virus mixture was then added to confluent monolayers of MDCK cells seeded in 96-well plates, incubated for 15 minutes at 4°C and then transferred to 37°C in a 5% CO_2 atmosphere for 45 minutes. After inoculation, the serum-virus supernatant was discarded and fresh Viral Production-Serum-Free Media (Invitrogen, CA, USA) supplemented with antibiotics/antimycotics and 1 μg/ml TPCK-trypsin (Invitrogen, CA, USA) added. Plates were then incubated at 37°C in 5% CO_2 atmosphere for 3 days and 50 μL of the supernatant collected and examined for presence of virus by hemagglutination assay.

For hemagglutination assay, 50 μL of sample cell culture supernatant or allantoic fluid (positive control) was mixed with 50 μL 1% cRBCs, incubated for 45 minutes at room temperature and wells examined for presence or absence of hemagglutination activity. Presence of HA activity indicated presence of virus and lack of neutralization by the serum sample tested.

2.2.8. Statistical analysis

Samples were categorized by virus, route of inoculation, and dosage. Two-paired T-tests were used to evaluate mean differences in IgY levels at all time points tested, viral shedding, and HI titers between groups. Spearman correlation coefficients were used to compare PR8 M2e-specific IgY with H5N3 M2e-specific IgY levels. A p-value <0.05 was considered statistically significant. Standard deviations for group means were calculated at 95% confidence. Statistical analysis was performed using JMP 9.0.0 Pro.

2.3. Results

2.3.1. M2e-specific IgY antibodies were produced in subcutaneous E2S1-M2e experimental groups

Thresholds for negative antibody production were calculated based on mean plus 3 times the standard deviation of the uninoculated control group. Antibody levels greater than the threshold were considered positive for antibody production. As shown in Fig. 2., all parental Sindbis E2S1 vaccinated and unvaccinated control chickens were negative for PR8 M2e-specific IgY antibodies at all time points before challenge regardless of dose and route of inoculation. Chickens vaccinated subcutaneously with E2S1-M2e at both high and low doses developed statistically significant levels of PR8 M2e-specific antibodies as early as 1-week post-initial vaccination compared to the uninoculated control.

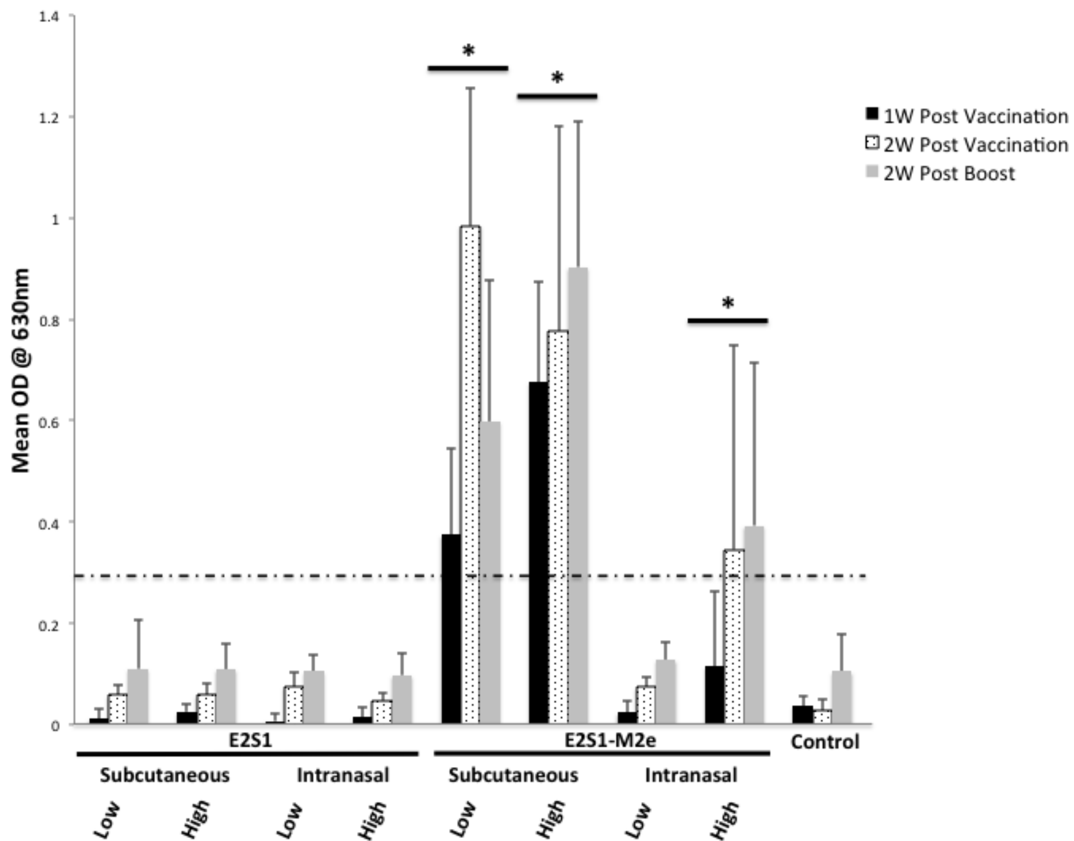

Fig. 2. Pre-challenge PR8 M2e-specific IgY ELISA results. Mean group M2e-specific IgY levels (OD @ 630 nm) are reported for each vaccination group with standard deviations at: 1 week (1W) post-vaccination, 2 weeks (2W) post vaccination and 2 weeks post boost. The threshold was calculated as mean plus 3 × the standard deviation of the uninoculated control group. Statistically different groups, p-value < 0.05 based on paired t-tests, are denoted with (*).

Furthermore, the high dose intranasally vaccinated E2S1-M2e group also developed statistically significant levels of M2e-specific antibodies vaccination, but to a lesser degree than the subcutaneously inoculated experimental groups and only at 2-weeks post-vaccination and 2-weeks post-challenge. The low dose intranasal experimental group remained negative for M2e-specific antibodies at all times tested pre-challenge.

2.3.2. M2e-specific IgA antibody levels were not detected

Tracheal washes collected pre-challenge (2-weeks post-boost) and post-challenge were assayed for M2e-specific IgA antibodies by ELISA. The IgA ELISA used for screening tested positive for ability to bind and detect chicken IgA antibodies. Results for tracheal wash IgA are reported as OD values at 630nm. As seen in Fig. 3, OD values for in all groups, were negative of M2e-specific IgA.

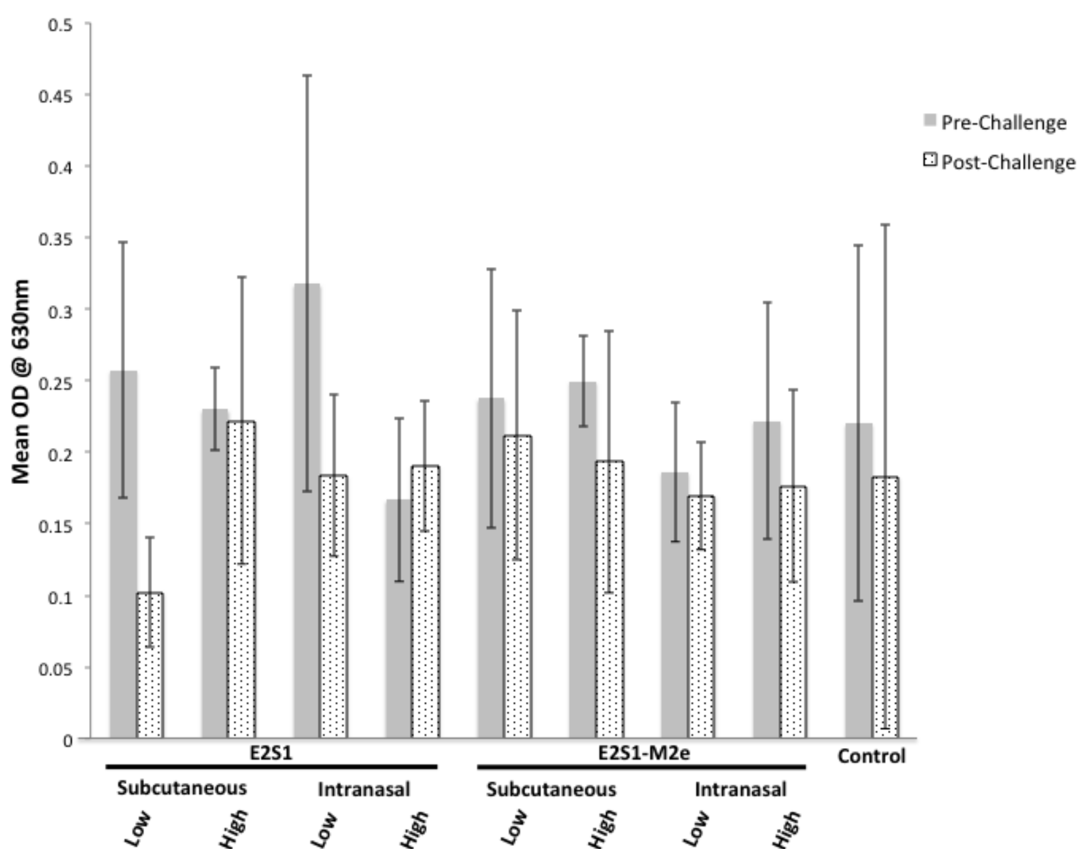

Fig. 3. Pre- and post-challenge PR8 M2e-specfic IgA ELISA results. ELISA results for M2e-specfic IgA in pre-challenge and post-challenge tracheal washes are reported as mean OD values at 630nm with standard deviations for each experimental group.

Upon sample concentration using centrifugal microconcentrators (Sartorius Stedim Biotech, Aubagne Cedex, France), sample OD values remained negative (data not shown). Serum samples pre- and post-challenge were also screened for M2e-specific IgA antibodies by ELISA. Based on OD values at 630nm, samples were also negative for M2e-specific IgA (data not shown). No further IgA analysis was performed.

2.3.3. M2e-specific IgY antibody levels significantly increased in groups with preexisting M2e-specific IgY antibodies after challenge

Two weeks after challenge with low pathogenic H5N3 AIV, chickens were bled and serum tested for PR8 M2e-specific IgY antibodies by ELISA. All groups, including parental Sindbis vaccinated and uninoculated control, developed PR8 M2e-specific antibodies after challenge with low pathogenic H5N3 AIV as determined by ELISA. This was expected as the challenge AIV itself contains M2 protein. A significant boost in M2e-specific antibody production was observed in the subcutaneously vaccinated groups (low and high dose) as well as the high dose intranasally inoculated group upon challenge. These groups developed statistically significantly higher antibody titers after challenge than naïve groups (Fig. 4).

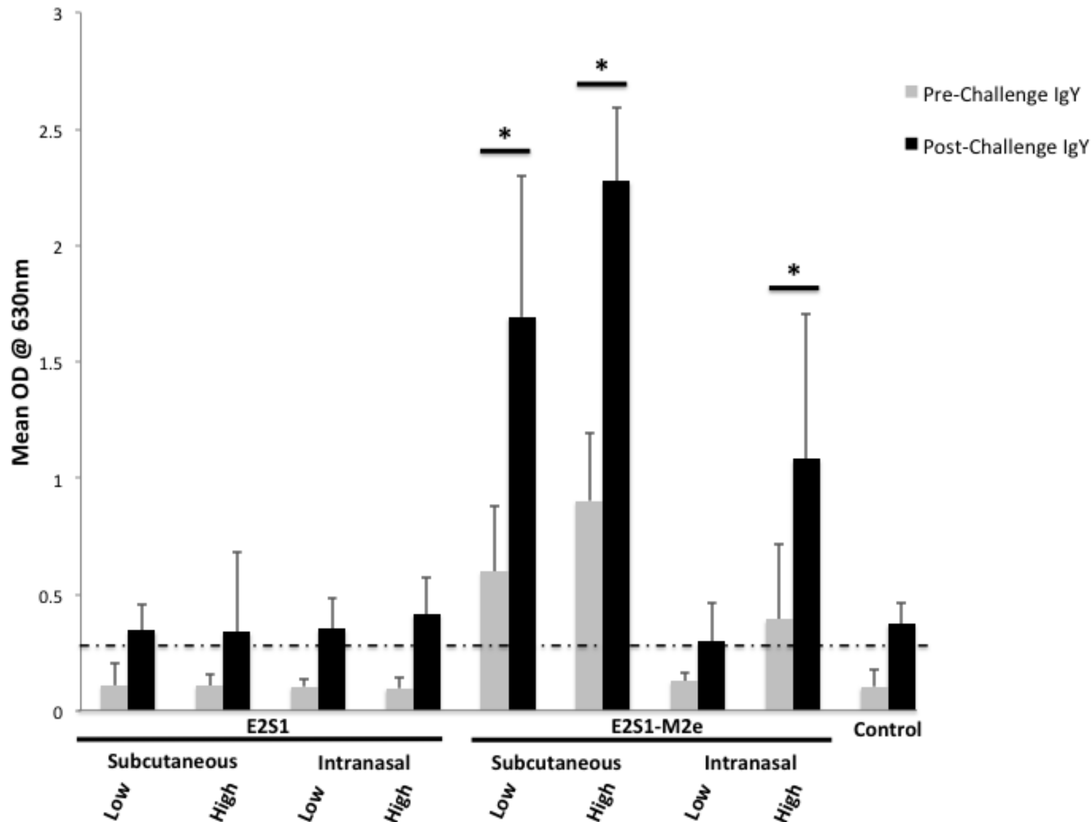

Fig. 4. PR8 M2e-specific IgY levels pre- and post-challenge with low pathogenic H5N3 AIV. M2e-specific IgY antibody levels are reported as mean group OD values at 630nm for each group at both time points. Threshold is calculated as mean plus 3 × standard deviation of the pre-challenge values of the uninoculated control group. Error bars represent standard deviation. Mean statistical differences based on paired t-tests with a p-value < 0.05 and are denoted with (*).

2.3.4. Challenge viral titers were similar in all groups

To examine vaccine efficacy, tracheal swabs were collected 4 days after challenge and viral shedding measured by real-time RT-PCR using the AgPath-ID^{TM} AIV-M Reagent Kit based on influenza virus matrix gene. Despite high levels of M2e-specific antibodies in both subcutaneously vaccinated E2S1-M2e groups, there were no significant differences in viral shedding between the groups (Fig. 5).

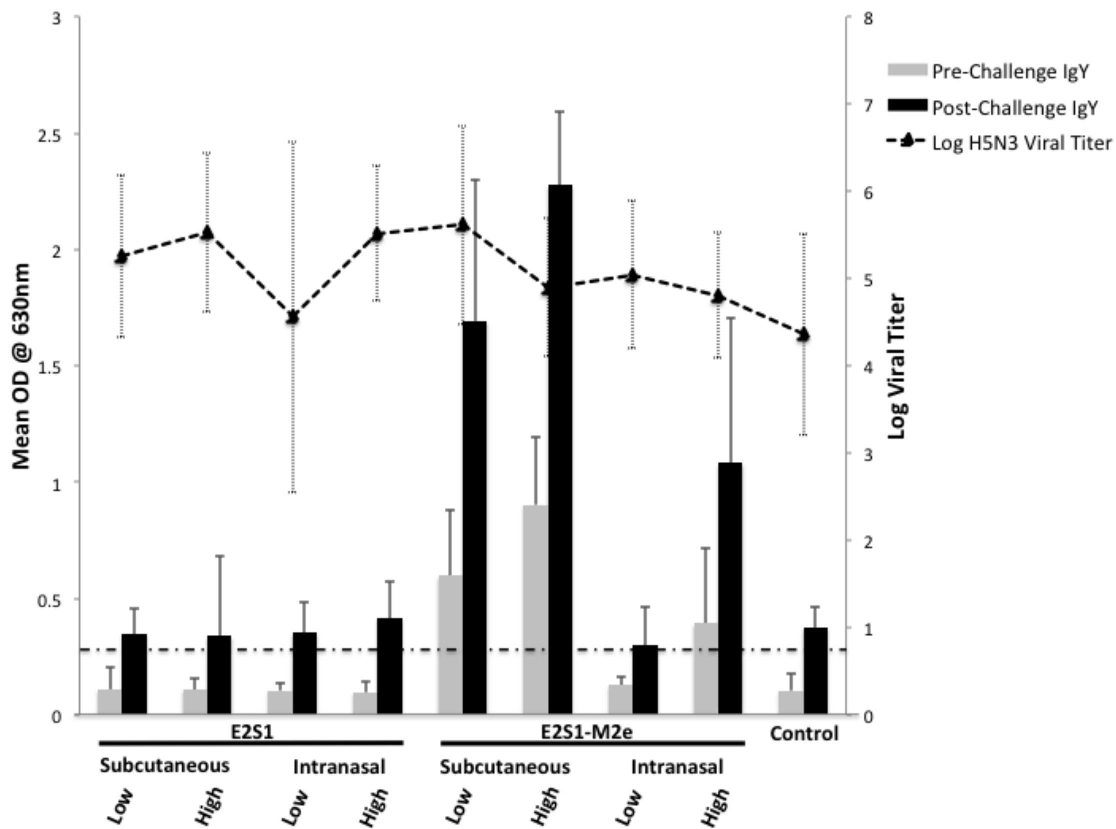

Fig. 5. Pre- and post-challenge PR8 M2e-specific IgY levels in comparison to challenge virus shedding. Mean group M2e-specific IgY levels are reported for each group pre-challenge (2 weeks post-boost) and post-challenge. Threshold was calculated as mean plus 3 × standard deviation of the uninoculated control group and shown as the line at the bottom of the graph. The overlaid line above the bar graph represents mean H5N3 virus titer of each group 4 days post-challenge and is reported as log viral titers with standard deviations. Mean statistical significance analysis of H5N3 AIV viral shedding titers based on paired t-tests with p-values < 0.05.

2.3.5. Challenge H5N3 M2e-specific IgY antibodies were detected in pre- and post-challenge serum samples

Cross reactivity of vaccine M2e specific IgY antibodies with M2e peptide from H5N3 challenge virus was examined on post-boost and post-challenge serum samples by

ELISA. As seen in Fig. 6, vaccine-induced antibodies recognizing the M2e peptide from H5N3 challenge virus were detected in pre-challenge serum for groups with preexisting M2e-specific antibody levels from E2S1-M2e vaccination; these levels were statistically higher than control groups. As expected, in groups with negative PR8 M2e-specific IgY antibodies levels post-boost, H5N3 M2e peptide binding antibodies were not detected.

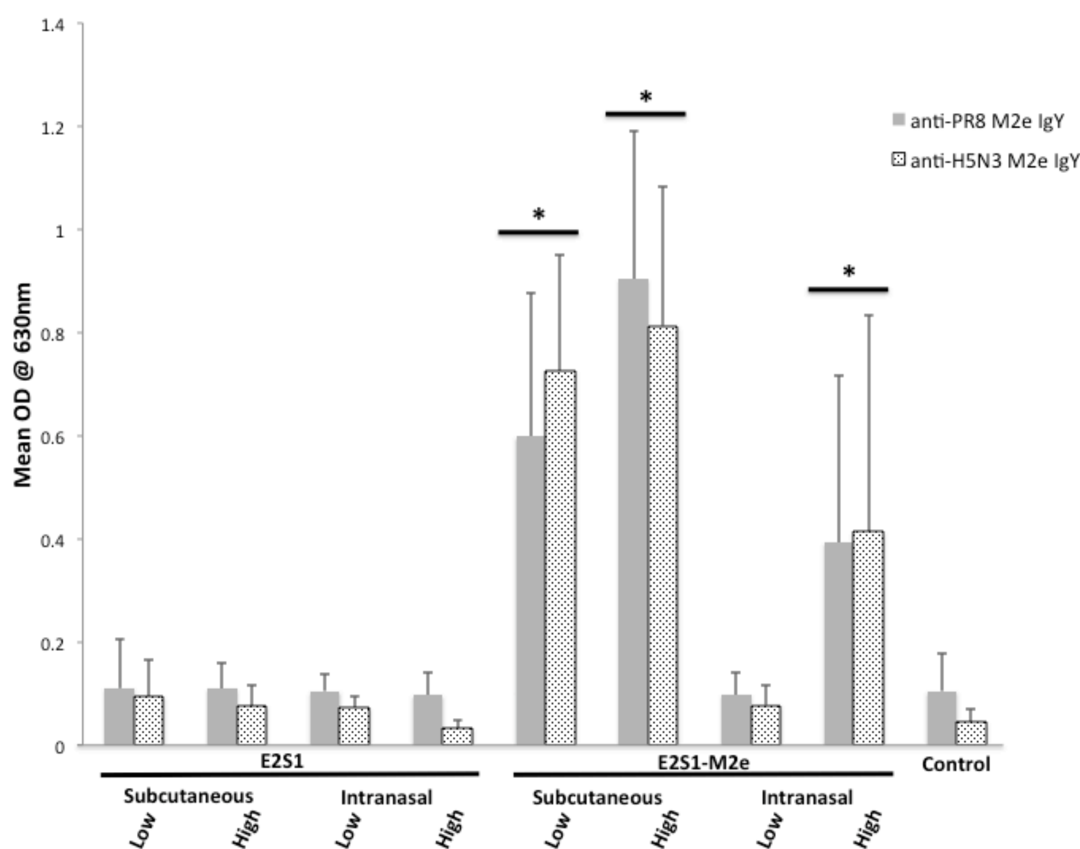

Fig. 6. M2e-specific IgY cross reactivity to H5N3 M2e peptide pre-challenge. Mean group M2e-specific IgY levels detected against PR8 and H5N3 M2e peptides are reported for each group pre-challenge. Paired t-tests and Spearman's correlation coefficients with p-values < 0.05 are considered significant. Statistically significant different means are denoted as (*).

Although the H5N3 M2e-specific IgY antibodies levels detected were not as high as the vaccine M2e-specific IgY antibody levels after challenge, the overall group M2e-specific antibody levels detected against H5N3 M2e correlate to the PR8 vaccine M2e-specific antibodies both before and after challenge (Fig. 7).

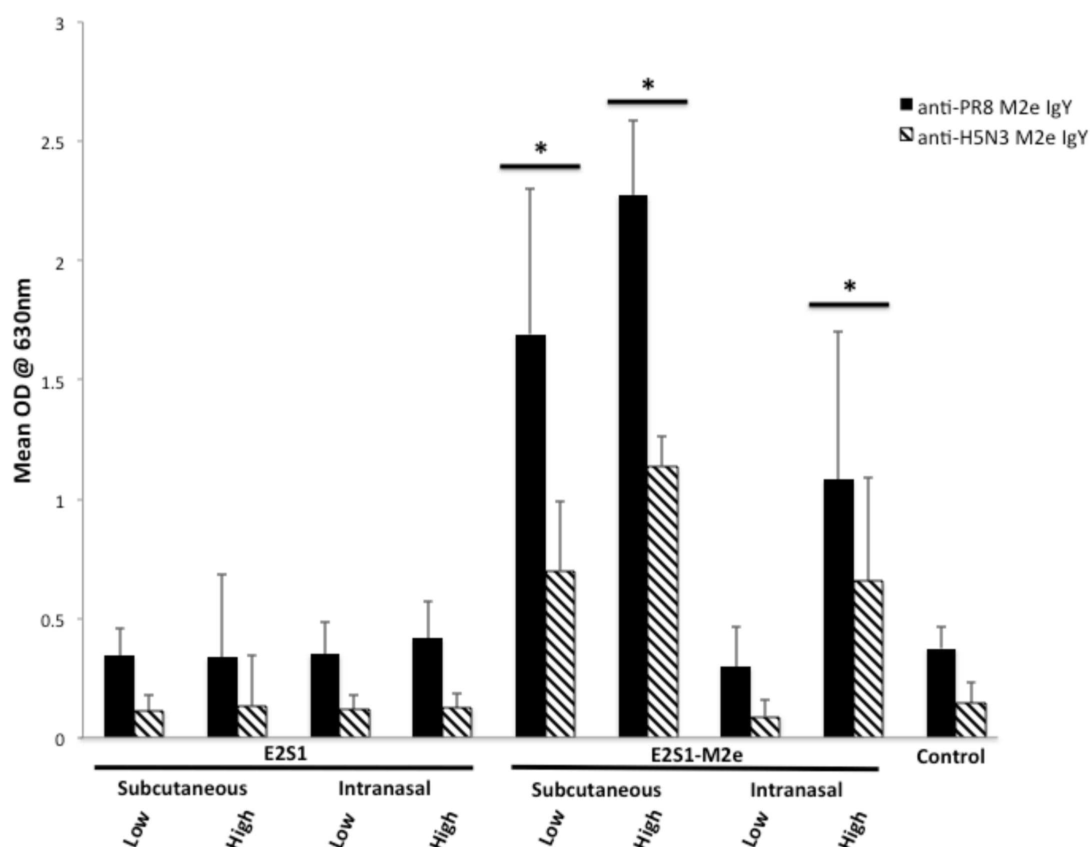

Fig. 7. M2e-specific IgY cross reactivity to H5N3 M2e peptide post-challenge. Mean group M2e-specific IgY levels detected against PR8 and H5N3 M2e peptides are reported for each group post-challenge. Paired t-tests and Spearman's correlation coefficients with p-values < 0.05 are considered significant. Statistically significant different means are denoted as (*).

Correlation between pre-challenge M2e-specific IgY antibodies detected against the vaccine PR8 M2e and H5N3 M2e peptide was statistically significant (p-value <0.0001). Correlation for post-challenge IgY levels was also statistically significant, with a p-value of 0.0025. The antibody detection levels for the H5N3 M2e peptide was consistently lower than the PR8 M2e antibody levels, even in the parental E2S1 vaccinated and uninoculated control groups (Fig. 7). Low detection sensitivity is suspected for the H5N3 M2e ELISA assay, as the mouse monoclonal 14C2 M2e-specific antibody control OD values were approximately halved in this assay compared to the PR8 M2e ELISA. Groups without pre-existing PR8 M2e-specific antibody levels before challenge should produce higher antibody levels against the H5N3 M2e after challenge.

2.3.6. Challenge H5N3 AIV successfully infected chickens and induced HA-specific antibodies production

HI assays were performed on post-challenge serum to test for successful infection of H5N3 challenge virus and production of HA-specific antibodies. Post-challenge serum was able to bind H5N9 virus in a HI assay and inhibit hemagglutination activity of the virus, verifying that AIV was able to infect and replicate in the birds and all birds developed H5-subtype specific antibodies (Fig. 8). No statistical differences in HI titers between were observed amongst groups.

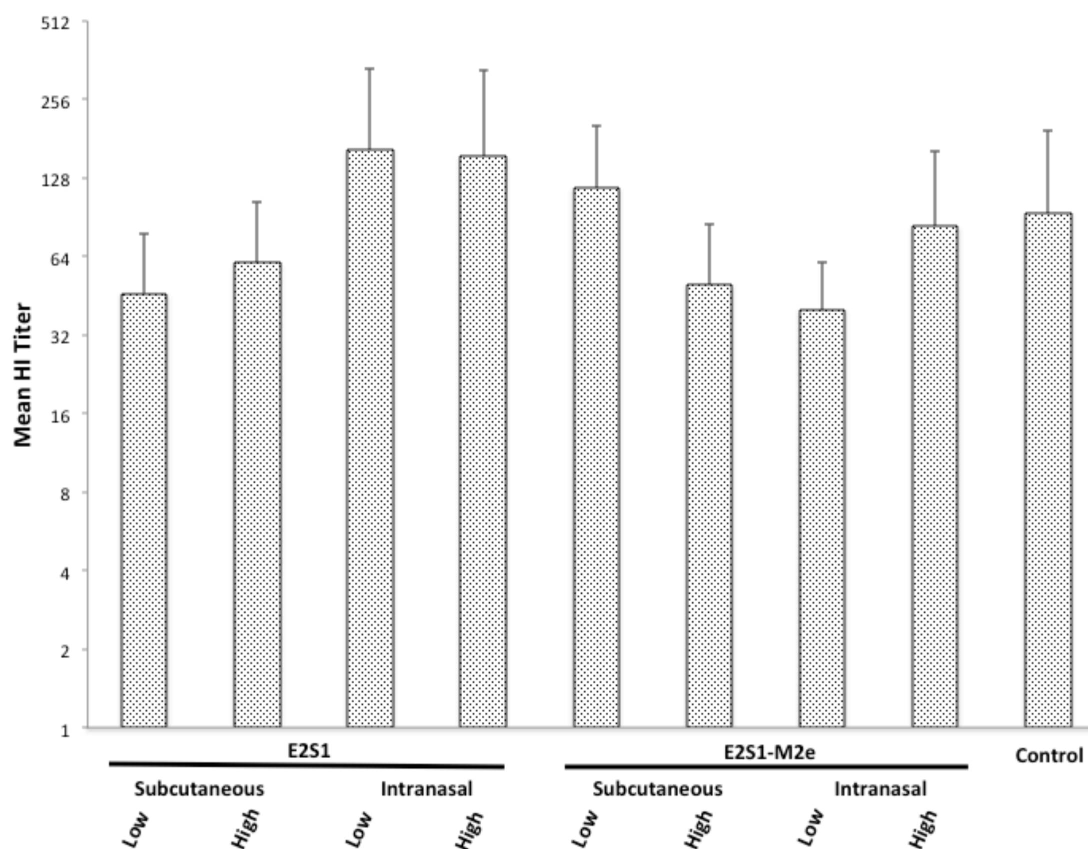

Fig. 8. Hemagglutination inhibition (HI) titers of post-challenge serum. Post-challenge serum was diluted by 2-fold serial dilution up to 1:256 and tested for inhibiting antibodies. HI titer is reported as the highest dilution at which hemagglutination inhibition occurs. Mean group HI titers are reported with standard deviations (8-9 chickens per group). Mean statistical differences based on paired t-tests p-values < 0.05.

2.3.7. M2e-specific IgY antibodies pre-challenge did not neutralize AIV in cell culture

Microneuralization assays using H5N9 AIV and MDCK cells were performed on pre- and post-challenge serum to test if M2e-specific IgY antibodies were able to neutralize AIV. Both monoclonal mouse IgG against M2e and positive control H5N9 chicken serum were able to neutralize the virus at all dilutions tested. As expected, pre-vaccination serum did not neutralize the virus as chickens were not exposed to the

vaccine or avian influenza. Pre-challenge serum samples from the subcutaneously vaccinated E2S1-M2e group with the highest OD values, containing only M2e-specific IgY antibodies against influenza, were unable to neutralize the H5N9 virus (data not shown). As expected, post-challenge serum samples, containing M2e-specific as well as HA-specific antibodies, were able to neutralize the virus at all dilutions tested.

2.4. Discussion

The use of AIV vaccination, which has not been routinely practiced by the United States in the past, is quickly becoming a recommended tool to prevent major losses to the poultry industry during AIV outbreaks (17). Due to international trade implications, the use of traditional inactivated AIV vaccines are not feasible in the United States as infected birds cannot be distinguishable from vaccinated birds serologically. Although countries in which avian influenza is endemic uses vaccination as a method to control AIV in domestic poultry, the traditional inactivated vaccines developed are subtype specific (25, 85) and requires reformulation, manufacturing, and production when new strains of AIV arise.

The objective of this study was to determine whether the E2S1-M2e virus, a Sindbis virus vector expressing the PR8 influenza M2e peptide, was a potential candidate for a universal DIVA vaccine against AIV by inducing M2e-specific antibody production and providing protection upon challenge with low pathogenic H5N3 AIV in chickens. Targeting the influenza M2e peptide enables possible cross-protection between different influenza A subtypes due to M2e's highly conserved sequence (50). Expressing influenza M2e on a heterologous virus generates the potential for a universal

vaccine with DIVA characteristics, making differentiation between infected and vaccinated birds possible (14, 26). The currently developed HA-based fowlpox, NDV, and HVT DIVA vaccines require reformulation and production as new HA subtypes of AIV circulate (13, 38). In contrast to these fowlpox and NDV DIVA vaccines, the successful development of an M2e-based DIVA vaccine would potentially provide universal immunity against multiple type A influenza strains, and therefore not require reformulation for each new outbreak.

Upon evaluation in SPF chickens, recombinant E2S1-M2e was able to induce high levels of M2e-specific IgY when inoculated by the subcutaneous route. Statistically significant levels of M2e-specific antibody production occurred in the high dose intranasally inoculated E2S1-M2e groups before challenge (Fig. 3), but levels were not as high as subcutaneously inoculated groups. This may be due to the difference in cell trophism between Sindbis virus, which is naturally transmitted by mosquitos intravenously, and AIV, which is transmitted by mucosal secretions and naturally infects the respiratory and enteric tracts (40, 127). There is also less control over dosage with intranasal vaccination, as birds may swallow the vaccine virus instead of inhaling the vaccine into the lungs during inoculation. An alternative route of inoculation would be to intravenously inject the vaccine, as Sindbis is naturally introduced into the host system through this route by mosquitos. Although AIV replicates well in the respiratory tract, considering the Sindbis vector delivery system requires intravenous inoculation, the vaccine may have had better efficacy had the inoculation route been based on the vector delivery system's replication requirements. The difficulty with intravenous

inoculation is that it would require individual handling of birds and trained personnel. Due to the extensive amount of chickens in a commercial setting, individual vaccination, especially intravenous inoculation, would be too labor intensive and is not a viable option for the poultry industry.

The high levels of M2e-specific antibodies induced by both high and low dose subcutaneous or high dose intranasal inoculation with E2S1-M2e were not able to provide protection upon challenge with low pathogenic H5N3 AIV, measured by rRT-PCR of viral shedding 4-days post-challenge (Fig. 5), conflicting with previous reports using M2e as a vaccine target (61, 113). Although previously published systems used to vaccinate against influenza M2e peptide have had promising results (61, 134), the results obtained from this E2S1-M2e study is one of the few in which the M2e-specific antibodies were not able to reduce viral shedding during infection (47). Some studies have described protection only against certain pathogenic influenza strains (61), also conflicting with the notion that M2e can confer universal influenza A protection.

The M2e-specific antibodies induced by E2S1-M2e were not able to neutralize AIV *in vitro* based on microneutralization assays, which was consistent with previous reports as M2e-specific antibodies are not known for neutralization (34, 48, 131). Most studies claim M2e-specific antibodies induce protection by opsonizing infected cells expressing the M2e peptide to recruit phagocytosis or cytotoxicity of the entire infected cell (34, 51). Although M2e-based vaccines do not prevent influenza infection from occurring, M2e-specific antibodies have shown to significantly reduce viral shedding, lessening the severity and spread of the disease (134). Differing to these reports, the

mouse monoclonal 14C2 M2e-specific antibody control used was able to neutralize the H5N9 AIV in the microneutralization assay (data not shown). A recent study have revealed M2e-specific antibodies may have some neutralization capability (1), stating that currently used neutralization assays do not allow adequate time for these antibodies to bind the virus since influenza virus expresses very low levels of M2 on its surface. The neutralization ability reported from this study was quite low despite the increased incubation times, requiring high concentration of the collected mouse serum (1). In this sense, M2e-based vaccines cannot replace inactivated HA-subtype specific vaccines to prevent occurrence of influenza infections. Multiple influenza epitope vaccines may be an alternative strategy for vaccination which would allow production of neutralizing antibodies by including the influenza HA protein (82) or induction of cytotoxic T cells with the use of influenza NP protein epitope (33). Vaccination of M2e in conjugation with other influenza proteins will increase immune responses while M2e provides universality and reduces viral shedding. M2e-specific antibodies induce strong Th2 responses, which may help strengthen cytotoxic T lymphocyte responses induced by the other influenza epitopes overall, generating both a strong humoral and cell-mediated immune response.

On closer inspection of the M2e peptide amino acid sequences used in this study, the PR8 M2e peptide (SLLTEVETPIRNEWGCRCNGSSD) used in the development of E2S1-M2e had a five amino acid difference from the low pathogenic H5N3 AIV M2e sequence (SLLTEVETP**T**RN**G**W**E**C**K**CN**D**SSD) used for challenge. The PR8 M2e peptide used in the E2S1-M2e virus was also of mouse-adapted human influenza origin,

while the challenge virus used in this chicken trial was an avian influenza virus. Earlier studies have used same origin M2e peptides for vaccination and challenge, i.e. vaccination with human influenza M2e peptide and challenge with human influenza, and the same with avian influenza studies (113, 128, 134, 136). This disparity may have contributed to the inefficiency of the M2e-specific antibodies induced by the E2S1-M2e vaccine. Despite the 5 amino acid differences, the vaccine-induced M2e-specific antibodies recognized and bound the H5N3 M2e peptide before challenge in groups with existing vaccine-induced M2e-specific antibody levels. These results suggest some M2e-specific IgY antibody cross-reactivity between the human-origin vaccine PR8 M2e peptide and the challenge H5N3 avian influenza M2e peptide, supporting previous work that M2e-specific antibodies are cross-reactive in poultry (61, 134). That PR8 M2e-specific antibody levels detected were higher than the H5N3 M2e-specific antibodies levels, which can be attributed to less sensitivity of the H5N3 M3e ELISA assay. Less sensitivity was determined by the halved OD values detected for mouse monoclonal 14C2 M2e-specific antibody control in the H5N3 M2e ELISA. Although these antibodies were cross-reactive, viral titers of the experimental vaccinated groups were not statistically different from the uninoculated control group (Fig. 5), suggesting the antibody-dependent cytotoxicity mechanism did not transpire (34, 51) or a strong Th2 response was lacking. Based on this study's results, the E2S1-M2e vector vaccine would not provide universal protection against AIV type A viruses in chickens.

For this study, the genetic differences within the M2e regions may not allow for cross-protection between influenza A viruses. M2e is more conserved amongst human

influenza strains than avian influenza strains, which may not permit the use of M2e peptide as a target for universal vaccination against avian influenza (65). With this in mind, human M2e-based vaccines against avian influenza may not be effective due to the less conserved nature of AIV's M2e peptide. Twenty-one new M2e variants have been found with the majority of the mutations at the center of the peptide (122). This needs to be taken into consideration during M2e universal vaccine development as M2e sequence changes may affect immunity. To further test the worth of M2e as a target for universal vaccination, another recombinant E2S1-M2e using an M2e peptide identical to the M2e of the avian influenza challenge virus should be developed to test for direct protection. If identical M2e peptides are necessary to provide protection against AIV based on reduced viral shedding, M2e may not be a viable target for universal type A vaccination.

3. SUMMARY

Chickens were vaccinated with parental E2S1 Sindbis virus or recombinant E2S1-M2e virus vector as a potential universal DIVA vaccine against avian influenza A. Vaccination with E2S1-M2e vector vaccine induced production of vaccine M2e-specific IgY antibodies when subcutaneously inoculated at both dosages. The intranasally inoculated E2S1-M2e group was able to produce some vaccine-induced M2e-speicific antibodies, but only at high dose. Upon challenge with low pathogenic H5N3 AIV, no significant difference in viral shedding was observed between any of the groups. HI results show the H5N3 challenge virus was able to successfully infect the chickens and induce the production of HA-specific antibodies. High M2e-specific IgY antibodies from pre-challenge subcutaneously-inoculated serum samples were not able to neutralize avian influenza virus upon microneutralization assay. Vaccine M2e-specific IgA production in all bird groups were negative at both pre- and post-challenge time points. Challenge H5N3 AIV M2e-specific IgY antibodies were detected in groups with pre-existing vaccine M2e-specific IgY antibodies before challenge, suggesting M2e-antibody cross-reactivity. All groups developed H5N3 M2e-specific IgY antibodies after challenge with H5N3 AIV, but groups with pre-existing M2e-specific antibody levels developed higher levels of both PR8 and H5N2 M2e-specific antibodies. Although the E2S1-M2e vaccine vector is able to induce the production of M2e-specific IgY antibodies capable of cross-reacting in the chicken when subcutaneously inoculated, these antibodies are unable to neutralize AIV or provide protection upon challenge with low pathogenic H5N3 AIV.

REFERENCES

1. Adler-Moore, J., M. Munoz, H. Kim, J. Romero, T. Tumpey, H. Zeng, C. Petro, W. Ernst, S. Kosina, G. Jimenez, and G. Fujii. Characterization of the murine Th2 response to immunization with liposomal M2e influenza vaccine. Vaccine 29:4460-4468. 2011.

2. Alexander, D.J. A review of avian influenza in different bird species. Vet. Microbiol. 74:3-13. 2000.

3. [APHIS] Animal and Plant Health Inspection Service. Avian influenza diagnostics and testing [Internet]. USDA, 2008 [Cited 2012 Dec]. Available from: http://www.aphis.usda.gov/animal_health/animal_dis_spec/poultry

4. Avellaneda, G., E. Mundt, C.W. Lee, S. Jadhao, and D.L. Suarez. Differentiation of infected and vaccinated animals (DIVA) using the NS1 protein of avian influenza virus. Avian Dis. 54:278-286. 2010.

5. Avellaneda, G., M.J. Sylte, C.W. Lee, and D.L. Suarez. A heterologous neuraminidase subtype strategy for the differentiation of infected and vaccinated animals (DIVA) for avian influenza virus using an alternative neuraminidase inhibition test. Avian Dis. 54:272-277. 2010.

6. Balasuriya, U.B., H.W. Heidner, N.L. Davis, H.M. Wagner, P.J. Hullinger, J.F. Hedges, J.C. Williams, R.E. Johnston, W. David Wilson, I.K. Liu, and N. James MacLachlan. Alphavirus replicon particles expressing the two major envelope proteins of equine arteritis virus induce high level protection against challenge with virulent virus in vaccinated horses. Vaccine 20:1609-1617. 2002.

7. Beard, C.W. Turkey influenza vaccination. Vet. Rec. 108:545. 1981.

8. BiondVaX. One for all: The universal flu vaccine [Internet]. Biondvax, Ness Ziona, Isreal. 2011 [Cited 2011 Nov]. Available from: http://www.biondvax.com

9. Black, R.A., P.A. Rota, N. Gorodkova, H.D. Klenk, and A.P. Kendal. Antibody response to the M2 protein of influenza A virus expressed in insect cells. J. Gen. Virol. 74:143-146. 1993.

10. Brahmakshatriya, V.R., B. Lupiani, and S.M. Reddy. Characterization and evaluation of avian influenza NS1 mutant virus as a potential live and killed DIVA (differentiating between infected and vaccinated animals) vaccine for chickens. Vaccine 28:2388-2396. 2010.

11. Brugh, M. Highly pathogenic virus recovered from chickens infected with mildly pathogenic 1986 isolates of H5N2 avian influenza virus. Avian Dis. 32:695-703. 1988.

12. Brugh, M., and M.L. Perdue. Emergence of highly pathogenic virus during selective chicken passage of the prototype mildly pathogenic chicken/Pennsylvania/83 (H5N2) influenza virus. Avian Dis. 35:824-833. 1991.

13. Bublot, M., R.J. Manvell, W. Shell, and I.H. Brown. High level of protection induced by two fowlpox vector vaccines against a highly pathogenic avian influenza H5N1 challenge in specific-pathogen-free chickens. Avian Dis. 54:257-261. 2010.

14. Bublot, M., N. Pritchard, D.E. Swayne, P. Selleck, K. Karaca, D.L. Suarez, J.C. Audonnet, and T.R. Mickle. Development and use of fowlpox vectored vaccines for avian influenza. Ann N Y Acad. Sci. 1081:193-201. 2006.

15. Capua, I., and D.J. Alexander. Avian influenza: recent developments. Avian Pathol. 33:393-404. 2004.

16. Capua, I., and G. Cattoli. Diagnosing avian influenza infection in vaccinated populations by systems for differentiating infected from vaccinated animals (DIVA). Dev. Biol. 130:137-143. 2007.

17. Capua, I., and S. Marangon. Control of avian influenza infections in poultry with emphasis on vaccination. Expert Rev. Anti Infect Ther. 4:751-757. 2006.

18. Capua, I., and S. Marangon. Vaccination policy applied for the control of avian influenza in Italy. Dev. Biol. 114:213-219. 2003.

19. Capua, I., F. Mutinelli, S. Marangon, and D.J. Alexander. H7N1 avian influenza in Italy (1999 to 2000) in intensively reared chickens and turkeys. Avian Pathol. 29:537-543. 2000.

20. Capua, I., C. Terregino, G. Cattoli, F. Mutinelli, and J.F. Rodriguez. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol. 32:47-55. 2003.

21. Cauthen, A.N., D.E. Swayne, M.J. Sekellick, P.I. Marcus, and D.L. Suarez. Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J. Virol. 81:1838-1847. 2007.

22. Centanni, E.a.S., E. La peste aviaria I & II. Communicazione fatta all'accademia delle scienze mediche e naturali de Ferrara. 1901.

23. Ceva. Ceva develops Vectormune HVT-AIV vaccine to combat avian influenza. In: 8th International Symposium on Avian Influenza. Ceva, London, England. p 1. 2012.

24. Ceva. Summary of study on the efficacy of five avian influenza vaccination regimens under Egyptian field condition in commercial broiler chickens carrying maternally derived antibody (MDA) to H5N1 AIV. In: 8th International Symposium on Avian Influenza. Ceva, London, England. pp 49-54. 2012.

25. Chen, H. Avian influenza vaccination: the experience in China. Revue scientifique et technique 28:267-274. 2009.

26. Chen, H.Y., Y.H. Shang, H.X. Yao, B.A. Cui, H.Y. Zhang, Z.X. Wang, Y.D. Wang, A.J. Chao, and T.Y. Duan. Immune responses of chickens inoculated with a recombinant fowlpox vaccine coexpressing HA of H9N2 avain influenza virus and chicken IL-18. Antiviral Res. 91:50-56. 2011.

27. Choi, Y.K., J.H. Lee, G. Erickson, S.M. Goyal, H.S. Joo, R.G. Webster, and R.J. Webby. H3N2 influenza virus transmission from swine to turkeys, United States. Emerg. Infect. Dis. 10:2156-2160. 2004.

28. Davison, S., R.J. Eckroade, and A.F. Ziegler. A review of the 1996-98 nonpathogenic H7N2 avian influenza outbreak in Pennsylvania. Avian Dis. 47:823-827. 2003.

29. De Filette, M., W. Fiers, W. Martens, A. Birkett, A. Ramne, B. Lowenadler, N. Lycke, W.M. Jou, and X. Saelens. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. Vaccine 24:6597-6601. 2006.

30. De Filette, M., T. Ysenbaert, K. Roose, M. Schotsaert, S. Roels, E. Goossens, B. Schepens, W. Fiers, and X. Saelens. Antiserum against the conserved nine amino acid N-terminal peptide of influenza A virus matrix protein 2 is not immunoprotective. J. Gen. Virol. 92:301-306. 2011.

31. De Vriese, J., Y. Gardin, V. Palya, K.M. Dorsey, B. Lambrecht, S. Van Borm, and T. van den Berg. Summary of Efficacy of a rHVT-AIV vector vaccine in broilers with passive immunity to HVT and AIV, against challenge with H5N1 HPAIV. In: 8th International Symposium on Avian Influenza. Ceva, London, England. pp 17-24. 2012.

32. De Vriese, J., M. Steensels, V. Palya, Y. Gardin, K.M. Dorsey, B. Lambrecht, S. Van Borm, and T. van den Berg. Passive protection afforded by maternally-derived antibodies in chickens and the antibodies' interference with the protection elicited by avian influenza-inactivated vaccines in progeny. Avian Dis. 54:246-252. 2010.

33. Dynavax. Pipeline flu vaccine [Internet]. Dynavax Technologies, Berkeley, CA. 2011 [Cited 2012 Jan]. Available from: http://www.dynavax.com/flu.html

34. El Bakkouri, K., F. Descamps, M. De Filette, A. Smet, E. Festjens, A. Birkett, N. Van Rooijen, S. Verbeek, W. Fiers, and X. Saelens. Universal vaccine based on

ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J. Immunol. 186:1022-1031. 2011.

35. Fu, T.M., D.C. Freed, M.S. Horton, J. Fan, M.P. Citron, J.G. Joyce, V.M. Garsky, D.R. Casimiro, Q. Zhao, J.W. Shiver, and X. Liang. Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. J. Virol. 385:218-226. 2009.

36. Garcia, M., J.M. Crawford, J.W. Latimer, E. Rivera-Cruz, and M.L. Perdue. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J. Gen. Virol. 77:1493-1504. 1996.

37. Ge, J., G. Deng, Z. Wen, G. Tian, Y. Wang, J. Shi, X. Wang, Y. Li, S. Hu, Y. Jiang, C. Yang, K. Yu, Z. Bu, and H. Chen. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. J. Virol. 81:150-158. 2007.

38. Ge, J., G. Tian, X. Zeng, Y. Jiang, H. Chen, and Z. Bua. Generation and evaluation of a Newcastle disease virus-based H9 avian influenza live vaccine. Avian Dis. 54:294-296. 2010.

39. Gillim-Ross, L., and K. Subbarao. Emerging respiratory viruses: challenges and vaccine strategies. Clin Microbiol Rev 19:614-636. 2006.

40. Griffin, D.E. Alphaviruses. In: Fields Virology, 5 ed. D. Knipe, Howley, P., ed. Lippincott Williams & Wilkins, Philadelphia, PA. pp 1023-1067. 2007.

41. Gupta, P.K., S.S. Dahiya, P. Kumar, A. Rai, C.L. Patel, A.A. Sonwane, and M. Saini. Sindbis virus replicon-based DNA vaccine encoding Rabies virus glycoprotein elicits specific humoral and cellular immune response in dogs. Acta Virol. 53:83-88. 2009.

42. Hall, J.S., K.T. Bentler, G. Landolt, S.A. Elmore, R.B. Minnis, T.A. Campbell, S.C. Barras, J.J. Root, J. Pilon, K. Pabilonia, C. Driscoll, D. Slate, H. Sullivan, and R.G. McLean. Influenza infection in wild raccoons. Emerg. Infect. Dis. 14:1842-1848. 2008.

43. Halvorson, D., Capua, I., Cardona, C., Frame, D., Karunakaran, D., Marangon, S., Ortali, G., Roepke, D., and Woo-Ming, B. The economics of avian influenza control. In: Western Poultry Disease Conference. Sacramento, CA. p 1. 2003.

44. Halvorson, D.A. Prevention and management of avian influenza outbreaks: experiences from the United States of America. Rev. Sci. Tech. 28:359-369. 2009.

45. Halvorson, D.A., D.D. Frame, K.A.J. Friendshuh, and D.P. Shaw. Outbreaks of low pathogenicity avian influenza in U.S.A. Avian Dis. 47:36-46. 2003.

46. Heidner, H.W., and R.E. Johnston. The amino-terminal residue of Sindbis virus glycoprotein E2 influences virus maturation, specific infectivity for BHK cells, and virulence in mice. J. Virol. 68:8064-8070. 1994.

47. Heinen, P.P., F.A. Rijsewijk, E.A. de Boer-Luijtze, and A.T. Bianchi. Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. J. Gen. Virol. 83:1851-1859. 2002.

48. Hughey, P.G., P.C. Roberts, L.J. Holsinger, S.L. Zebedee, R.A. Lamb, and R.W. Compans. Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. J. Virol. 212:411-421. 1995.

49. Huleatt, J.W., V. Nakaar, P. Desai, Y. Huang, D. Hewitt, A. Jacobs, J. Tang, W. McDonald, L. Song, R.K. Evans, S. Umlauf, L. Tussey, and T.J. Powell. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 26:201-214. 2008.

50. Ito, T., O.T. Gorman, Y. Kawaoka, W.J. Bean, and R.G. Webster. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J. Virol. 65:5491-5498. 1991.

51. Jegerlehner, A., N. Schmitz, T. Storni, and M.F. Bachmann. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. J. Immunol. 172:5598-5605. 2004.

52. Jiao, P., G. Tian, Y. Li, G. Deng, Y. Jiang, C. Liu, W. Liu, Z. Bu, Y. Kawaoka, and H. Chen. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J. Virol. 82:1146-1154. 2008.

53. Kappes, M.A., M.R. Sandbulte, R. Platt, C. Wang, K.M. Lager, J.N. Henningson, A. Lorusso, A.L. Vincent, C.L. Loving, J.A. Roth, and M.E. Kehrli, Jr. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. Vaccine. 2011.

54. Karunakaran, D., V. Hinshaw, P. Poss, J. Newman, and D. Halvorson. Influenza A outbreaks in Minnesota turkeys due to subtype H10N7 and possible transmission by waterfowl. Avian Dis. 27:357-366. 1983.

55. Keawcharoen, J., D. van Riel, G. van Amerongen, T. Bestebroer, W.E. Beyer, R. van Lavieren, A.D. Osterhaus, R.A. Fouchier, and T. Kuiken. Wild ducks as long-

distance vectors of highly pathogenic avian influenza virus (H5N1). Emerg. Infect. Dis. 14:600-607. 2008.

56. Kinde, H., D.H. Read, B.M. Daft, M. Hammarlund, J. Moore, F. Uzal, J. Mukai, and P. Woolcock. The occurrence of avian influenza A subtype H6N2 in commercial layer flocks in Southern California (2000-02): clinicopathologic findings. Avian Dis. 47:1214-1218. 2003.

57. Klimstra, W.B., J.C. Williams, K.D. Ryman, and H.W. Heidner. Targeting Sindbis virus-based vectors to Fc receptor-positive cell types. J. Virol. 338:9-21. 2005.

58. Kuiken, T., E.C. Holmes, J. McCauley, G.F. Rimmelzwaan, C.S. Williams, and B.T. Grenfell. Host species barriers to influenza virus infections. Science 312:394-397. 2006.

59. Kumar, S.R., L. Deflube, M. Biswas, R. Shobana, and S. Elankumaran. Genetic characterization of swine influenza viruses (H3N2) isolated from Minnesota in 2006-2007. Virus Genes 43:161-176. 2011.

60. Lamb, R.A., S.L. Zebedee, and C.D. Richardson. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell 40:627-633. 1985.

61. Layton, S.L., D.R. Kapczynski, S. Higgins, J. Higgins, A.D. Wolfenden, K.A. Liljebjelke, W.G. Bottje, D. Swayne, L.R. Berghman, Y.M. Kwon, B.M. Hargis, and K. Cole. Vaccination of chickens with recombinant Salmonella expressing M2e and CD154 epitopes increases protection and decreases viral shedding after low pathogenic avian influenza challenge. Poult. Sci. 88:2244-2252. 2009.

62. Lee, C.W., and D.L. Suarez. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J. Virol. Methods 119:151-158. 2004.

63. Lee, C.W., and D.L. Suarez. Avian influenza virus: prospects for prevention and control by vaccination. Anim. Health Res. Rev. 6:1-15. 2005.

64. Li, Y., K. Reddy, S.M. Reid, W.J. Cox, I.H. Brown, P. Britton, V. Nair, and M. Iqbal. Recombinant herpesvirus of turkeys as a vector-based vaccine against highly pathogenic H7N1 avian influenza and Marek's disease. Vaccine 29:8257-8266. 2011.

65. Liu, W., P. Zou, J. Ding, Y. Lu, and Y.H. Chen. Sequence comparison between the extracellular domain of M2 protein human and avian influenza A virus provides new information for bivalent influenza vaccine design. Microbes Infect. 7:171-177. 2005.

66. Londt, B.Z., J. Banks, R. Gardner, W.J. Cox, and I.H. Brown. Induced increase in virulence of low virulence highly [corrected] pathogenic avian influenza by serial intracerebral passage in chickens. Avian Dis. 51:396-400. 2007.

67. Marangon, S., and L. Busani. The use of vaccination in poultry production. Revue scientifique et technique 26:265-274. 2007.

68. Mesonero, A., D.L. Suarez, E. van Santen, D.C. Tang, and H. Toro. Avian influenza in ovo vaccination with replication defective recombinant adenovirus in chickens: vaccine potency, antibody persistence, and maternal antibody transfer. Avian Dis. 55:285-292. 2011.

69. Mohanty, S., Dutta, S. Veterinary virology. Lea & Febiger, Philadelphia, PA. 1981.

70. Moore, T., and N. Morgan. Avian influenza: trade issues. In: CAST Commentary. C. Beard and B. Gardner, eds. Council for Agricultural Science and Technology, Ames, IA. p 1. 2006.

71. Moulthrop, J.I., and A. Langston. Report on avian influenza outbreak in Minnesota turkeys--1978. Comp Immunol Microbiol Infect Dis. 3:193-200. 1980.

72. [NIH] National Institutes of Health. Phase I safety and immunogenicity study of VAX102 [Flagellin.HuM2e] influenza vaccine in healthy adults [Internet]. 2011 [Cited 2011 Nov 14]. Available from: http://clinicaltrials.gov/ct2/show/NCT00603811

73. [NIH] National Institutes of Health. Safety study of recombinant M2e influenza-A vaccine in healthy adults (FLU-A) [Internet]. 2010 [Cited 2011 Nov 14]. Available from: http://clinicaltrials.gov/ct2/show/NCT00819013?term=Safety+Study+of+Recombinant+M2e+Influenza-A+Vaccine+in+Healthy+Adults&rank=1

74. [NPIP] National Poultry Improvement Plan. Animals and animal products: National Poultry Improvement Plan for commercial poultry [Internet]. National Archives and Records Administration, 2012 [Cited 2012 Feb 1]. Available from: http://www.gpo.gov/fdsys/pkg/CFR-2010-title9-vol1/xml/CFR-2010-title9-vol1-part146.xml

75. Nayak, B., S.N. Rout, S. Kumar, M.S. Khalil, M.M. Fouda, L.E. Ahmed, K.C. Earhart, D.R. Perez, P.L. Collins, and S.K. Samal. Immunization of chickens with Newcastle disease virus expressing H5 hemagglutinin protects against highly pathogenic H5N1 avian influenza viruses. PLoS One 4:e6509. 2009.

76. Neirynck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W.M. Jou, and W. Fiers. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat. Med. 5:1157-1163. 1999.

77. Nfon, C., Y. Berhane, S. Zhang, K. Handel, O. Labrecque, and J. Pasick. Molecular and antigenic characterization of triple-reassortant H3N2 swine influenza viruses isolated from pigs, turkey and quail in Canada. Transbound. Emerg. Dis. 58:394-401. 2011.

78. [OIE] Office International des Epizooties. Avian influenza [Internet]. OIE, Paris, France. 2011 [Cited 2012 Jan]. Available from: http://www.oie.int/international-standard-setting/terrestrial-code

79. [OIE] Office International des Epizooties. Prescribed and alternative diagnostic tests for OIE listed diseases [Internet]. OIE, Paris, France. 2011 [Cited 2012 Jan]. Available from: http://web.oie.int/eng/normes/mcode/en_titre_1.1.htm

80. Palese, P., Shaw, M. Orthomyxoviridae: The viruses and their replication. In: Fields Virology, 5 ed. D. Knipe, Howley, P., ed. Lippincott Williams & Wilkins, Philadelphia, PA. pp 1647-1689. 2007.

81. Pan, C.H., C.E. Greer, D. Hauer, H.S. Legg, E.Y. Lee, M.J. Bergen, B. Lau, R.J. Adams, J.M. Polo, and D.E. Griffin. A chimeric alphavirus replicon particle vaccine expressing the hemagglutinin and fusion proteins protects juvenile and infant rhesus macaques from measles. J. Virol. 84:3798-3807. 2010.

82. Park, K.S., Y.B. Seo, J.Y. Lee, S.J. Im, S.H. Seo, M.S. Song, Y.K. Choi, and Y.C. Sung. Complete protection against a H5N2 avian influenza virus by a DNA vaccine expressing a fusion protein of H1N1 HA and M2e. Vaccine 29:5481-5487. 2011.

83. Pelzel, A.M., B.J. McCluskey, and A.E. Scott. Review of the highly pathogenic avian influenza outbreak in Texas, 2004. J. Am. Vet. Med. Assoc. 228:1869-1875. 2006.

84. Perroncito, E. Epizoozia tifoide nei gallinacei. Annali Accad. Agri. Torino 21:87-126. 1878.

85. Peyre, M., H. Samaha, Y.J. Makonnen, A. Saad, A. Abd-Elnabi, S. Galal, T. Ettel, G. Dauphin, J. Lubroth, F. Roger, and J. Domenech. Avian influenza vaccination in Egypt: Limitations of the current strategy. J. Mol. Genet. Med. 3:198-204. 2009.

86. Qiao, C., Y. Jiang, G. Tian, X. Wang, C. Li, X. Xin, H. Chen, and K. Yu. Recombinant fowlpox virus vector-based vaccine completely protects chickens from H5N1 avian influenza virus. Antiviral Res. 81:234-238. 2009.

87. Rajagopal, S., and J. Treanor. Pandemic (avian) influenza. Semin. Respir. Crit. Care Med. 28:159-170. 2007.

88. Ramp, K., J. Veits, D. Deckers, M. Rudolf, C. Grund, T.C. Mettenleiter, and A. Romer-Oberdorfer. Coexpression of avian influenza virus H5 and N1 by recombinant

Newcastle disease virus and the impact on immune response in chickens. Avian Dis. 55:413-421. 2011.

89. Rauw, F., V. Palya, S. Van Borm, S. Welby, T. Tatar-Kis, Y. Gardin, K.M. Dorsey, M.M. Aly, M.K. Hassan, M.A. Soliman, B. Lambrecht, and T. van den Berg. Further evidence of antigenic drift and protective efficacy afforded by a recombinant HVT-H5 vaccine against challenge with two antigenically divergent Egyptian clade 2.2.1 HPAI H5N1 strains. Vaccine 29:2590-2600. 2011.

90. Reed, J.L., and Muench, H. A simple method of estimating fifty percent endpoints. Am. J. Epidemiol. 27:493-497. 1938.

91. Regan, W.M., and Prisloe, M. R. The economic impact of avian influenza on Connecticut's egg industry. In. Department of Economic and Community Development, Hartford, CT. 2003.

92. Saxena, S., S.S. Dahiya, A.A. Sonwane, C.L. Patel, M. Saini, A. Rai, and P.K. Gupta. A sindbis virus replicon-based DNA vaccine encoding the rabies virus glycoprotein elicits immune responses and complete protection in mice from lethal challenge. Vaccine 26:6592-6601. 2008.

93. Schafer, W. Verglieichender sero-immunologische Untersuchungen uber die Viren der Influenza und klassischen Geflugelpest. Z Naturf 10b:81-91. 1955.

94. Schroer, D., J. Veits, G. Keil, A. Romer-Oberdorfer, S. Weber, and T.C. Mettenleiter. Efficacy of Newcastle disease virus recombinant expressing avian influenza virus H6 hemagglutinin against Newcastle disease and low pathogenic avian influenza in chickens and turkeys. Avian Dis. 55:201-211. 2011.

95. Seo, S.H., E. Hoffmann, and R.G. Webster. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8:950-954. 2002.

96. Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka. Avian flu: influenza virus receptors in the human airway. Nature 440:435-436. 2006.

97. Siembieda, J.L., C.K. Johnson, C. Cardona, N. Anchell, N. Dao, W. Reisen, and W. Boyce. Influenza A viruses in wild birds of the Pacific flyway, 2005-2008. Vector Borne Zoonotic Dis. 10:793-800. 2010.

98. Singh, S., H. Toro, D.C. Tang, W.E. Briles, L.M. Yates, R.T. Kopulos, and E.W. Collisson. Non-replicating adenovirus vectors expressing avian influenza virus hemagglutinin and nucleocapsid proteins induce chicken specific effector, memory and effector memory CD8(+) T lymphocytes. Virology 405:62-69. 2010.

99. Smith, G.J., D. Vijaykrishna, J. Bahl, S.J. Lycett, M. Worobey, O.G. Pybus, S.K. Ma, C.L. Cheung, J. Raghwani, S. Bhatt, J.S. Peiris, Y. Guan, and A. Rambaut. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125. 2009.

100. Stubbs, E.L. Fowl pest. In: Diseases of poultry, 1st ed. H.E.a.D. Biester, L., ed. Iowa State College Press, Ames, IA. pp 493-502. 1943.

101. Suarez, D.L. Overview of avian influenza DIVA test strategies. Biologicals 33:221-226. 2005.

102. Swayne, D.E., J.R. Beck, and N. Kinney. Failure of a recombinant fowl poxvirus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox vaccine. Avian Dis. 44:132-137. 2000.

103. Swayne, D.E., J.R. Beck, and T.R. Mickle. Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. Avian Dis. 41:910-922. 1997.

104. Swayne, D.E., and D.L. Suarez. Current developments in avian influenza vaccines, including safety of vaccinated birds as food. Dev. Biol. 130:123-133. 2007.

105. Swayne, D.E., D.L. Suarez, S. Schultz-Cherry, T.M. Tumpey, D.J. King, T. Nakaya, P. Palese, and A. Garcia-Sastre. Recombinant paramyxovirus type 1-avian influenza-H7 virus as a vaccine for protection of chickens against influenza and Newcastle disease. Avian Dis. 47:1047-1050. 2003.

106. Swayne, D.E.a.D.A.H. Influenza. In: Diseases of poultry, 11 ed. Y.M. Saif, H.J. Barnes and J.R. Glisson, eds. Iowa State University Press, Ames, IA. pp 135-160. 2003.

107. Swayne, D.S., and B.L. Akey. Avian influenza control strategies in the United States of America. In: Avian influenza: prevention and control. R.S.S.a.G. Koch, ed. Springer, Dordrecht. pp 113-130. 2005.

108. Talon, J., M. Salvatore, R.E. O'Neill, Y. Nakaya, H. Zheng, T. Muster, A. Garcia-Sastre, and P. Palese. Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach. Proc Natl Acad Sci U S A 97:4309-4314. 2000.

109. Tang, Y., C.W. Lee, Y. Zhang, D.A. Senne, R. Dearth, B. Byrum, D.R. Perez, D.L. Suarez, and Y.M. Saif. Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis. 49:207-213. 2005.

110. Thomas, J.M., W.B. Klimstra, K.D. Ryman, and H.W. Heidner. Sindbis virus vectors designed to express a foreign protein as a cleavable component of the viral structural polyprotein. J. Virol. 77:5598-5606. 2003.

111. Thomas, J.M., S.T. Moen, B.T. Gnade, D.A. Vargas-Inchaustegui, S.M. Foltz, G. Suarez, H.W. Heidner, R. Konig, A.K. Chopra, and J.W. Peterson. Recombinant Sindbis virus vectors designed to express protective antigen of Bacillus anthracis protect animals from anthrax and display synergy with ciprofloxacin. Clin. Vaccine Immunol. 16:1696-1699. 2009.

112. Thomssen, R. Live attenuated versus killed virus vaccines. Monogr. Allergy 9:155-176. 1975.

113. Tompkins, S.M., Z.S. Zhao, C.Y. Lo, J.A. Misplon, T. Liu, Z. Ye, R.J. Hogan, Z. Wu, K.A. Benton, T.M. Tumpey, and S.L. Epstein. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg. Infect. Dis. 13:426-435. 2007.

114. Toro, H., D.L. Suarez, D.C. Tang, F.W. van Ginkel, and C. Breedlovea. Avian influenza mucosal vaccination in chickens with replication-defective recombinant adenovirus vaccine. Avian Dis. 55:43-47. 2011.

115. Treanor, J.J., E.L. Tierney, S.L. Zebedee, R.A. Lamb, and B.R. Murphy. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J. Virol. 64:1375-1377. 1990.

116. Tumpey, T.M., R. Alvarez, D.E. Swayne, and D.L. Suarez. Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J. Clin. Microbiol. 43:676-683. 2005.

117. Turley, C.B., R.E. Rupp, C. Johnson, D.N. Taylor, J. Wolfson, L. Tussey, U. Kavita, L. Stanberry, and A. Shaw. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. Vaccine 29:5145-5152. 2011.

118. [USDA] United States Department of Agriculture. Animal health monitoring and surveillance: Poultry monitoring and surveillance [Internet]. USDA, 2012 [Cited 2012 Jan]. Available from: http://www.aphis.usda.gov/vs/nahss/poultry/index.htm

119. United States Poultry and Egg Association. Industry economic data, consumption, exports, processing, production [Internet]. Tucker, GA. 2011 [Cited 2011 Dec]. Available from: http://www.uspoultry.org/economic_data

120. Veits, J., D. Wiesner, W. Fuchs, B. Hoffmann, H. Granzow, E. Starick, E. Mundt, H. Schirrmeier, T. Mebatsion, T.C. Mettenleiter, and A. Romer-Oberdorfer.

Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. Proc Natl Acad Sci U S A 103:8197-8202. 2006.

121. Walker, J.A., and Y. Kawaoka. Importance of conserved amino acids at the cleavage site of the haemagglutinin of a virulent avian influenza A virus. J. Gen. Virol. 74:311-314. 1993.

122. Wang, Y., L. Zhou, H. Shi, H. Xu, H. Yao, X.G. Xi, T. Toyoda, X. Wang, and T. Wang. Monoclonal antibody recognizing SLLTEVET epitope of M2 protein potently inhibited the replication of influenza A viruses in MDCK cells. Biochem. Biophys. Res. Commun. 385:118-122. 2009.

123. Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers, and Y. Kawaoka. Evolution and ecology of influenza A viruses. Microbiol. Rev. 56:152-179. 1992.

124. Wise, H.M., A. Foeglein, J. Sun, R.M. Dalton, S. Patel, W. Howard, E.C. Anderson, W.S. Barclay, and P. Digard. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J. Virol. 83:8021-8031. 2009.

125. Wolf, A.I., K. Mozdzanowska, K.L. Williams, D. Singer, M. Richter, R. Hoffmann, A.J. Caton, L. Otvos, and J. Erikson. Vaccination with M2e-based multiple antigenic peptides: characterization of the B cell response and protection efficacy in inbred and outbred mice. PLoS One 6:e28445. 2011.

126. Woolcock, P.R., D.L. Suarez, and D. Kuney. Low-pathogenicity avian influenza virus (H6N2) in chickens in California, 2000-02. Avian Dis. 47:872-881. 2003.

127. Wright, P., Neumann, G., Kawaoka, Y. Orthomyxoviruses. In: Fields Virology, 5 ed. D. Knipe, Howley, P., ed. Lippincott Williams & Wilkins, Philadelphia, PA. 2007.

128. Wu, F., X.Y. Yuan, W.S. Huang, and Y.H. Chen. Heterosubtypic protection conferred by combined vaccination with M2e peptide and split influenza vaccine. Vaccine 27:6095-6101. 2009.

129. Xu, R., R. McBride, J.C. Paulson, C.F. Basler, and I.A. Wilson. Structure, receptor binding, and antigenicity of influenza virus hemagglutinins from the 1957 H2N2 pandemic. J. Virol. 84:1715-1721. 2010.

130. Yassine, H.M., C.W. Lee, and Y.M. Saif. Interspecies Transmission of Influenza A Viruses Between Swine and Poultry. Curr. Top. Microbiol. Immunol. 2011.

131. Zebedee, S.L., and R.A. Lamb. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J. Virol. 62:2762-2772. 1988.

132. Zebedee, S.L., C.D. Richardson, and R.A. Lamb. Characterization of the influenza virus M2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. J. Virol. 56:502-511. 1985.

133. Zhang, G.G., D.X. Li, H.H. Zhang, Y.M. Zeng, and L. Chen. Enhancement of mucosal immune response against the M2eHBc+ antigen in mice with the fusion expression products of LTB and M2eHBc+ through mucosal immunization route. Vet. Res. Commun. 33:735-747. 2009.

134. Zhang, X., M. Liu, C. Liu, J. Du, W. Shi, E. Sun, H. Li, J. Li, and Y. Zhang. Vaccination with Different M2e Epitope Densities Confers Partial Protection against H5N1 Influenza A Virus Challenge in Chickens. Intervirology. 2011.

135. Zhou, C., L. Zhou, and Y.H. Chen. Immunization with high epitope density of M2e derived from 2009 pandemic H1N1 elicits protective immunity in mice. Vaccine Article in Press. 2012.

136. Zhou, D., T.L. Wu, M.O. Lasaro, B.P. Latimer, E.M. Parzych, A. Bian, Y. Li, H. Li, J. Erikson, Z. Xiang, and H.C. Ertl. A universal influenza A vaccine based on adenovirus expressing matrix-2 ectodomain and nucleoprotein protects mice from lethal challenge. Mol. Ther. 18:2182-2189. 2010.

137. Ziegler, A.F., S. Davison, H. Acland, and R.J. Eckroade. Characteristics of H7N2 (nonpathogenic) avian influenza virus infections in commercial layers, in Pennsylvania, 1997-98. Avian Dis. 43:142-149. 1999.