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Evaluation of Protective Efficacy of Viral Vector Based Swine Influenza Vaccine and a Method for B Cell Culture for Monoclonal Antibody Generation

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EVALUATION OF PROTECTIVE EFFICACY OF VIRAL VECTOR BASED SWINE INFLUENZA VACCINE AND A METHOD FOR B CELL CULTURE FOR MONOCLONAL ANTIBODY GENERATION

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Influenza A virus of the swine (IAV-S) is an economically important swine pathogen that has the potential to spread to humans, thus posing an ongoing public health concern due to its zoonotic potential. Hemagglutinin (HA), the most abundant viral envelope protein, is known to be the key protective antigen. Anti-HA antibodies alone, have been shown to prevent IAV infection. In this study we evaluated the feasibility of a recombinant tri segmented Pichinde virus (PICV) as a viral vector to deliver IAV-S hemagglutinin antigen in pigs. Four groups of weaned pigs (T01-04) were immunized twice with PBS, rPICV-GFP as a vector control, rPICV-H3 and H3-protein, respectively, at day 0 and 21 followed by an intra-tracheal challenge with a wild-type H3N2 IAV-S strain on day 42. T03 and T04 groups seroconverted and exhibited high titers of plasma neutralizing antibodies after vaccination. Post challenge infection, undetectable or minimal levels of influenza virus shedding, and lung lesions were observed in T03 and T04 groups whereas high levels of shedding and lung lesions were observed in T01 and T02 groups. Overall, both rPICV-H3 and H3-protein were able to confer solid protection in pigs and no significant difference in terms of protection was observed between these two vaccine types. These results provide the basis for the continuous development of the rPICV to use as a potential viral vector vaccine in pigs.

While the vaccines act as a preventive measurement against infectious diseases, monoclonal antibodies (mAb) act as a therapeutic tool for treating already infected subjects. In the second part of the study, a new method for gene isolation and in vitro mAb generation was evaluated. Unlike the conventional hybridoma method where all the B cells are immortalized to continuously express antibodies, in this method antigen specific murine B cells were sorted and cultured in vitro to isolate variable region genes of the antibody and then cloned in a plasmid vector to transfect mammalian cells for expressing that particular antibody. By using this method, a complete functional murine mAb was successfully generated that was detect and bind specifically to the antigen. In future the same method can be employed to generate mAbs from swine and other systems as well.

Dedicated to my beloved PARENTS and BROTHER

For all their sacrifices, enormous love, encouragement, continuous support and for always having my back.

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ABBREVIATIONS

IAV-S	Influenza A Virus-Swine
PICV	Pichinde virus
RNA	Ribonucleic acid
H3	Hemagglutinin protein from H3N2 IAV-S subtype-3
GFP	Green fluorescent protein
PRDC	porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
PCV	Porcine circovirus
NSP7	PRRSV non-structural protein 7
IgG	Immunoglobulin isotype G
FACS	Fluorescence assisted cell sorting
IL	Interleukin

cDNA	complementary Deoxyribonucleic acid
CD40L	Cluster of differentiation 40 ligands
HEK	Human embryonic kidney cells
HA	hemagglutinin protein
NA	Neuraminidase protein
M	Matrix protein
PB	Polymerase basic
NP	Nucleoprotein
mRNA	messenger RNA
S	Small
Z	Zinc finger domain
GPC	Glycoprotein
ORF	Open reading frame
GOI	Gene of interest
BHK-21	Baby hamster kidney cell line
CTL	Cytotoxic T lymphocytes
TARV	Turkey arthritis reovirus
PFU	Plaque forming units

EDTA	Ethylenediamine tetraacetic acid
DPC	Days post Challenge
PK-15	Porcine kidney cell line
FBS	Fetal bovine serum
P/S	Penicillin/streptomycin
PAM	Porcine alveolar macrophages
RPMI	Roswell Park Memorial Institute
MDCK	Madin-Darby Canine Kidney cell line
BSA	Bovine serum albumin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MCS	Multiple cloning site
MOI	Multiplicity of infection
PBS	Phosphate buffered saline
hr	Hour
IFA	Immunofluorescence
BALF	Bronchioalveolar lavage fluid
Mins	Minutes
RDE	Receptor destroying enzyme

qPCR	Quantitative PCR
RBC	Red blood cells
CT	Cycle threshold
H2O2	Hydrogen peroxide
DPV	Days post vaccination
ELISA	Enzyme linked immunosorbent assay
RT-PCR	reverse transcription – polymerase chain reaction
qPCR	Quantitative PCR
MVA	Modified vaccinia virus Ankara PRVPseudorabies virus
rAd	recombinant Adenovirus
SIV	Swine influenza virus
L	Large

GENERAL INTRODUCTION

According to the National Animal Health Monitoring System (NAHMS), respiratory diseases are the most important cause of economical loss to swine producers. The primary agents involved in the porcine respiratory disease complex (PRDC) includes Porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus, porcine circovirus (PCV) and other opportunistic bacterial agents Mycoplasma hyopneumoniae, Bordetella bronchiseptica, and Actinobacillus pleuropneumoniae. Influenza A virus of the swine (IAV-S) and PRRSV are known to be the two major viral contributors to PRDC. Influenza virus carries a segmented, negative sense RNA genome. The viral RNA-dependent RNA polymerase lacks exonuclease proof reading capacity leading to high genomic mutation rates. Vaccination is the main tool to control the spread of the disease. This thesis consists of two chapters. In chapter 2, the main objective was to evaluate the immunogenicity and protective efficacy of a recombinant pichinde virus as a live viral vector to deliver the hemagglutinin antigen of IAV-S in pigs. In chapter 3, the main objective was to optimize a method for cloning and expression of functional IgG antibodies in mouse model from antigen specific B cells. Briefly, B cells specific to NSP7 protein of PRRSV were sorted using

fluorophore tagged NSP7 protein. The cells were expanded ex vivo by co-culture with CD40L expressing feeder cells in the medium supplemented with recombinant murine IL-2 and IL-21. Total RNA was extracted from the B cell clones that were expressing antibody against NSP7 in the supernatant. The variable regions of the heavy and light chains were PCR amplified and cloned into expression vectors containing constant regions of the mouse IgG genes. The resulting expression plasmids were transfected to HEK-293-T cells to produce a NSP7-specific monoclonal antibody.

CHAPTER 1: LITERATURE REVIEW

INFLUENZA A VIRUS

1.1 Structure of the virus

Swine Influenza is a very important respiratory disease of the pigs. The causative agent of the disease, Influenza A virus, belongs to the Orthomyxoviridae family [1]. The virus genome consists of negative sense RNA genome. A total of eight RNA segments encodes 11 proteins [1]. The host derived, lipid envelope membrane surrounding the virus particle presents two main antigenic determinants of the virus; hemagglutinin (HA), neuraminidase (NA) and a third integral membrane protein M2 [2]. Just beneath the lipid bilayer lies a layer of matrix protein M1. The core of the virus particle consists of eight RNA segments complexed with polymerase proteins PB1, PB2 and PA and a nucleoprotein (NP) that has RNA-dependent RNA polymerase activity [3]. The negative sense genomic RNA is noninfectious and needs viral transcriptase to first transcribe into the positive sense complimentary mRNA for protein translation [4].

1.2 Hemagglutinin protein

The surface of IAV consists of two important antigenic determinants: the glycoprotein HA and NA; of which HA plays a crucial role in mediating viral entry into the cells [5]. HA is a trimeric glycoprotein composed of a membrane-distal globular head domain and a membrane proximal stalk domain [6, 7]. HA recognizes and binds to the sialic acid residues present on the surface of the host cells. Following viral endocytosis, in the acidic condition HA undergoes a conformational change that fuses the viral membrane and the endosomal membrane of the host cell [8]. This phenomenon facilitates the virus genome release. Anti-HA antibodies neutralize virus infection by blocking the viral attachment to the host cells [9, 10]. However, the major challenge for IAV

vaccine development is that HA antigenic epitopes change constantly, which allows the virus to escape the pre-existing antibodies induced by previous vaccination [11].

1.3 Genomic mutation

Unlike many other RNA viruses, the influenza virus genome replication and transcription are carried out in the host cell's nucleus [12]. The viral polymerase of influenza virus lacks exonuclease proofreading capacity, making the virus a dynamic population with high gene mutation rates [3]. Every mutation that helps the virus to evade the host's immune response may be positively selected and passed on to the next generation. There can be mainly two different mechanisms by which the influenza virus can evolve: point mutation (antigenic drift) and gene reassortment (antigenic shift). In rare cases recombination in the genomic RNA can also take place [13, 14].

Point Mutation (antigenic drift) is defined as accumulation of random mutations at the antigenic sites in the surface glycoproteins: either HA and/or NA leading to gradual evolution of the virus. Hence, previously acquired antibodies can no longer interact with the antigenic domain allowing the virus to escape from the pre-existing immunity [13, 15].

Reassortment, or antigenic shift, occurs when the cells are simultaneously infected with two genotypically different influenza viruses [16]. Due to the segmented nature of the viral genome, exchange of RNA segments between the two viruses might occur which will result in an entirely new virus variant [17]. The pandemic of 1957 was caused by reassortant virus containing HA, NA and PB1 from avian origin and remaining genes from the 1918 strain, and 1968 pandemic was caused by the reassortant virus containing HA and PB1 from avian origin and remaining genes from the 1918 strain [18].

1.4 IAV-S variants

Isolated from pigs, first in 1931 [19], the classical H1N1 IAV-S was found to have evolved from the 1918 Spanish flu human strain [20]. In 1998, a new triple reassortant H3N2 virus was introduced into the swine population that contained HA, NA, and PB1 RNA segments from seasonal human H3N2 influenza, PB2 and PA from avian influenza, and NP, M and NS from the classical H1N1 virus [21]. These triple-reassortant viruses started co-circulating with the classical H1N1 viruses and resulted in reassortant viruses of new lineages of H1N1 and H1N2 [22, 23]. The introduction of H1N1pdm09 into the swine population lead to another lineage of reassortant viruses between this subtype and the locally endemic strains [24, 25].

Currently there are three major subtypes that are co-circulating in the swine population worldwide: H1N1, H1N2, and H3N2 [20]. Though it might be just three different subtypes, the origin, genetic background, and the antigenic properties of these IAV-S vary considerably. Among all the co-circulating IAV-S strains in the swine population of the United States, there are at least 10 antigenically different HA lineages- classical swine lineage: H1α, H1β, H1γ [26, 27]; human seasonal H1 lineage: H1δ1, H1δ2 [28]; H1N1pdm09 lineage [29]; and H3 cluster I-IV [21, 30, 31]. The subsequent increment in the generation of new antigenic variants and the global rise in number of infected populations indicates the importance of controlling the disease [32]. The most effective way of controlling IAV-S is by vaccinating the animals to prevent them from getting infected [33-35].

1.5 Current vaccine strategies

Most current vaccines are based on the whole inactivated viruses [34, 36, 37]. A few of them are based on replicating live attenuated viruses (LAIV) or RNA target delivery platform.

Whole inactivated viruses (WIV) are usually locally produced, and they contain a mix of different strains circulating within that region [38]. They are administered intramuscularly along with an adjuvant [39, 40]. Whole inactivated viruses are safe to use and provide robust protection in case of homologous infection. But they fail to show any efficacy in case of heterologous infection [41, 42]. It has been shown that the immunization of pigs with WIV in the presence of maternally derived antibodies, enhances the disease pathology in case the pigs get infected by an antigenically or genetically distinct virus strain [43].

Live attenuated viruses (LAIV) are replicating influenza viruses with a mutation or a set of mutations in the genome that reduces their pathogenicity, one example is the truncation of H3N2 IAV-S NS1 protein that impairs the virus from antagonizing the IFN response and they replicate poorly in the pig cells. LAIV provides good antibody as well as cellular immune response in the vaccinated subject. Even in the case of heterologous infection the LAIV vaccination can provide partial protection [43-46]. But we cannot eliminate the fact that these are replicating viruses and they can reassort with the cocirculating virus to become virulent any time [47].

The third type of vaccine depends on a foreign RNA particle to deliver the HA antigen gene to the dendritic cells in the host system for effective antigen presentation and induction of immune response (Sequivity, Merck). The veterinarians help in analyzing and selecting the strain that needs to be included for a specific locality or a particular farm, based on that the HA genes of those selective strains are inserted into the RNA particle.

Other than these three licensed commercial vaccines, there are many ongoing research studies, that are exploring other modes of antigen delivery for the development of vaccines for pigs.

1.6 Vector based Vaccine strategies against Influenza viruses

To date, several reported studies have been performed by using different virus-based vectors as a carrier for delivering influenza antigens [15, 48-55]. Viral vectors have been shown to induce both cell mediated and humoral immune responses [55]. The following paragraphs will summarize different types of viral vectors that have been used to deliver IAV antigens.

MVA is a highly attenuated strain of vaccinia virus that was serially passaged in chicken embryo fibroblasts and lost its ability to replicate in any other cell type than avian cells [56-59]. Unlike wild-type vaccinia strain, the MVA has lost the capacity to evade host's immune system [56, 58]. One advantage of the MVA vector is that it can accommodate the insertion of multiple foreign antigenic genes thus making it suitable for developing multivalent vaccines [56]. Recombinant MVA expressing the HA genes from H1N1pdm09 tested for their immunogenicity have shown to induce virus neutralizing antibody and T cell response, protection against homologous challenge, and some level of protection even in case of heterosubtypic challenge with other H1N1 strain [60]. Even though swine influenza viruses were used to test the vector, most of the studies have been performed in mice and ferrets [60].

Orf virus (ORFV), a member of Poxviridae family, is a ubiquitous virus that causes mucocutaneous infections in sheep, goats, and wild ruminants [61, 62]. Several features like restricted host range [63], ability to induce both humoral and cellular immune response, its cellular tropism (restricted to the skin keratinocytes) [62], lack of vector specific neutralizing antibodies, and its large genome size capable to of accommodating multiple gene of interests (GOI) makes it a potential vaccine delivery platform. The ORFV carrying HA or HA and NP gene together from H1N1 IAV-S were tested for their efficacy elicited robust antibody response in the pigs, and the HA-NP construct was shown to induce higher antibody as well as CD8+T cell response in the pigs.

Overall, there was complete protection against the homologous virus challenge. The serum isolated from the animals also showed cross neutralization activity against panel of IAV-S isolates belonging to the major genetic clades of current circulating virus in swine, demonstrating that the ORFV can be a potential vector for the swine system [64].

Alphavirus replicon particle is a propagation defective virus which enters inside the cells in the first round of infection and delivers the gene into the cytoplasm but cannot spread from one cell to another [65-67]. Alphavirus replicon particle vectored influenza virus vaccine is the first recombinant product to be approved for use as a vaccine in pigs in the United States [*Center for Veterinary Biologics*. Notice N. 17–01. 2017]. The vaccine is made of an attenuated Venezuelan-equine encephalitis virus, that is replication defective because of HA gene substitution from North American cluster-IV H3N2 in place of viral structural genes [68]. The viral structural genes are provided as a helper RNA. With the assistance from helper RNA, replicon particle RNA is efficiently packed and is indistinguishable from the native alphavirus particle [69]. The vaccine was administered in a prime-boost schedule with 2-3 weeks gap between each dose. The pigs showed reduced influenza virus RNA in the nasal swab and BALF samples, and considerable protection from pathological damage in case of homologous challenge but failed to show efficacy in the presence of maternally derived antibodies [67, 68].

PICHINDE VIRUS

2.1 General overview

Pichinde virus (PICV) is an enveloped RNA virus, belonging to the family of Arenaviridae [70]. PICV has a negative sense, bisegmented RNA genome, which is very well conserved among all the family members [71, 72]. Using a unique ambisense coding strategy, the two viral RNA segments, Large (L) and Small (S), encode for four viral proteins [73]. The L segment is ~7.2 kb in length and encodes for the RNA-dependent RNA polymerase (L polymerase) and the small ring finger protein Z. The S RNA segment encodes for glycoprotein (GPC) and nucleoprotein (NP) [72]. The Z protein is indicated to be involved in viral assembly, budding, and regulation of viral RNA synthesis [74]. The viral polymerase L protein is required for viral RNA transcription and translation in the cytoplasm of host cell [75, 76]. The post translationally activated proteins, derived from GPC, are known to mediate receptor binding and membrane fusion [77, 78]. The NP and Z protein have been shown to be a type I-IFN antagonists, contributing to modulation of host's innate immune responses [79, 80]. Even though rodents are the natural host for PICV, the virus can replicate in a wide range of hosts like macaques, humans, birds, and mouse.

2.2 Recombinant PICV as viral vectors

An attractive reason for selecting the recombinant pichinde virus as a vector is that they mainly target the antigen presenting cells that are macrophages and dendritic cells [81] in vivo. There have been no known cases of pathogenicity caused by the PICV in any other organism than the rodents, even though they can infect both human as well as porcine cells. A recombinant trisegmented PICV (rPICVtri) was generated to be used as a viral vector for vaccine development in which, the two S RNA segments were incorporated into the recombinant virus. S1 segment

carries the viral NP gene and a multiple cloning site for insertion of a gene of interest (GOI) [81]. Similarly, the S2 RNA segment carries the viral GPC gene and another multiple cloning site which allows the insertion of a second GOI. With this design, two different GOIs can be inserted into the triPCIV vector. The recombinant, trisegmented PICV replicates less efficiently in cell culture as it has smaller plaque size than its parental strain and yield about 1-1.5 log lower in viral titers [82]. But in terms of genetic stability, The triPICV seems to be working well, as the expression of GOI were maintained even after the virus was serially passaged in BHK-21 and Vero cells several times [83]. Importantly, the triPICV can deliver vaccine antigens in multiple animal species. In one of the studies, the HA and NP antigens of the IAV H1N1 was inserted into the triPICV and immunization of mice and guinea pigs with the triPICV-HA resulted in high titers of neutralizing antibody and CTL response. Mice injected with just a single dose of immunization were completely protected from a lethal challenge with homologous influenza virus strain [83]. The triPICV vector has also been tested in turkey. In this study, a triPICV expressing (TARV) antigen of Turkey Arthritis Reovirus was constructed, which when used to vaccinate turkeys, with two doses of triPICV vaccine at two-week interval displayed high titer of serum neutralizing antibody [84].

CHAPTER 2: EVALUATION OF PROTECTIVE EFFICACY OF A VIRAL VECTOR BASED SWINE INFLUENZA VACCINE

INTRODUCTION

IAV-S is a highly contagious, acute respiratory infection-causing pathogen that results in significant economic losses for the global pig productions [85]. IAV-S by itself is known to have high morbidity and low mortality rate (ranging from 1-4 %) [20], but in cases where opportunistic pathogens like the PRRSV, PCV, and different bacterial agents coinfect the pigs can result in significant enhanced mortality [86].

IAV genome is composed of eight negative sense segmented RNA. Two of these segments encode the immunogenically important surface protein HA and NA [87], required for viral attachment and release from the infected cells [12, 88, 89]. IAV carries its own RNA dependent RNA polymerase that lacks the exonuclease proof reading capacity making its genome prone to frequent variations [3, 15]. It can be minor changes due to point mutations like insertions or deletions in the antigen domain, referred to as antigenic drift or major antigenic change due to swapping of gene segments between two antigenically different viruses called antigenic shift [90]. Current subtypes of Influenza A virus of the swine circulating all over the world are H1N1, H3N2 and H1N2 [20]. Pigs consists of both sialic acid α2,3Gal (avian receptor) and sialic acid α2,6Gal (mammalian receptor) HA binding receptor that allows them to be infected by both the viruses [18] and thus making them susceptible to infection by both avian and human flu viruses [91]. Which means, when both the viruses infect an individual pig at the same time, genetic reassortment can take place giving rise to a novel strain with a broader host tropism [18, 32, 92]. There have been multiple cases where these viruses have jumped from the swine into the human population.

Repeated outbreaks rapidly spreading genetically, and antigenically distinct IAV-S poses an ongoing threat for swine production and public health.

The most effective measure to control IAV-S infection is by vaccinating the pigs to prevent the spread of the virus [34]. Most of the current vaccines are based on the whole inactivated viruses (WIV), whereas some of them are replicating modified live vaccine (MLV), or RNA vector expressing HA [15]. However, current vaccines are safe and provide good protection against homologous infection, but in case of heterologous infection the vaccine efficacy reduces significantly with low or no protection [39]. In certain cases, the WIV have been found to enhance the disease pathology if animal gets infected by an antigenically different strain known as vaccine associated enhanced respiratory disease (VAERD) [41, 93]. Though the vaccines available at present can limit disease progression up to certain level, they cannot consistently prevent virus shedding or transmission and provide protection against multiple strains of influenza virus [20]. Thus, there is an urgent need for evaluating other tools for vaccine development against the swine influenza A virus which either has the potential of being a vaccine production platform, that can be quicky updated with current circulating strains and/or is universal.

In this study we are evaluating a new pichinde virus (PICV) as a vector for IAV-S vaccine in the pigs. Pichinde virus belongs the family Arenaviridae. PICV is an enveloped virus with bisegmented, negative sense single-stranded RNA genome. Each genome segment (S and L) separated by a noncoding intergenic region, uses an ambisense coding strategy to express two polypeptides. While the S segment encodes GPC and N, the L segment encodes the L polymerase (viral RdRp) and Z protein. Although, PICV is naturally known to cause pathogenesis in rodents, in which antigen presenting cells- macrophages and dendritic cells are the primary targets, PICV is also permissive to other cell types like kidney cells, epithelial cells from a wide range of species

including macaques, humans, and pigs without causing any disease [18]. The wild type PICV, was modified to carry three gene segments with one L and two S RNA, where each S RNA segment can carry a gene of interest along with one of the viral genes. Which makes it capable of carrying two GOI altogether other than the four self-genes [16]. With all these advantageous features of pichinde virus: to target the antigen presenting cells, a better safety profile, lack of strong anti-vector immune response and antigen encoding capacity in such diverse range of host species [78, 84], we wanted to explore whether the recombinant PICV can serve as an efficient viral vector in swine too.

MATERIALS AND METHODS

1. Cells, Reagents and Viruses used

Baby hamster kidney (BHK-21) and Porcine kidney-15 (PK-15) cell line were cultured in complete Dulbecco's Modified Eagle Medium (DMEM, Gibco #12100-061) supplemented with 10% fetal bovine serum (FBS, SAFC Biosciences #12103C) and 1% of 100U Penicillin-Streptomycin (pen/strep, Sigma #P4333). Primary porcine alveolar macrophages (PAM) isolated and crypreserved from previous study pigs were cultured in complete Roswell Park Memorial Institute 1640 (RPMI-1640, Gibco #11835-0) supplemented with 10% FBS and 1% pen/strep. Madin-Darby Canine Kidney (MDCK) cell line was cultured in DMEM supplemented with 10% FBS, 1% pen/strep, 0.2% Bovine serum albumin (BSA, Sigma Cat. # A8412), and 25mM HEPES (Hyclone, Cat. # SH3023701). All the cell cultures and infections were incubated in the incubator at 37°C with 5% CO2, in a humidified condition.

Recombinant H3 protein from IAV-S strain H3N2 was expressed using the baculovirus expression system via a contract with GenScript. IAV-S H3N2 strain TX98 was obtained from USDA National Veterinary Services Laboratories (NVSL). The virus was propagated in the MDCK cells in infection medium containing DMEM supplemented with 1% pen/strep, 0.2% BSA, and 25mM HEPES and 1ug/ml of Trypsin-TPCK treated (Thermofisher #20233).

Recombinant Pichinde virus constructs expressing H3 antigen of the H3N2 strain TX98 and green fluorescent protein (herein designated as rPICV-H3 and rPICV-GFP, respectively) was generated by our collaborators Dr. Ly at University of Minnesota. The HA gene was inserted into both S1 and S2 segment of the recombinant, trisegmented PICV (Figure 2.1).

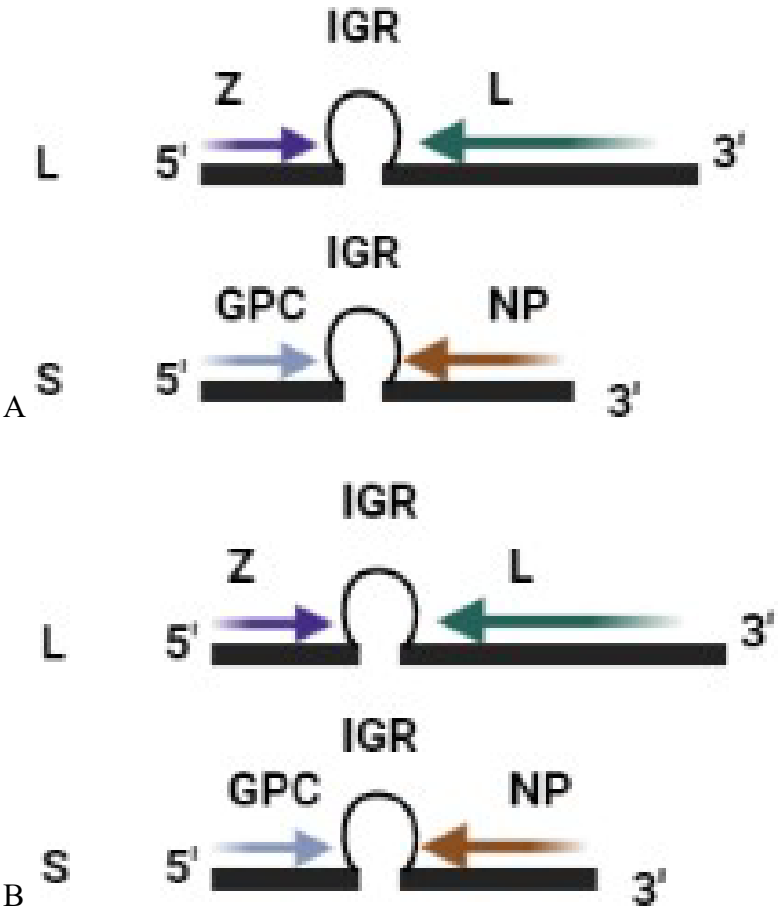


Figure 2.1. Genomic organization

(A) Large (L) and Small (S) RNA segments of wild type PICV carrying two genes on each segment in opposite orientation separated by an intergenic region. (B) The recombinant PICV construct with IAV-S subtype 3 hemagglutinin gene (H3), rPICV-H3 has GPC gene on S1 and NP on S2. The gene for IAV-S subtype 3 hemagglutinin (H3) antigen is inserted in MCS of both S1 and S2.

2. Detection of antigen expression in vitro

Antigen expression of rPICV-H3 and rPICV-GFP was evaluated in three different cell types: BHK-21, PK-15, and PAM. BHK-21 and PK-15 cells were seeded in 24-well plates at density of 1x105 cells per well in 500µl of complete DMEM. PAM cells were seeded in 24 well plates at density of 5x105 cells per well in 500µl of complete RPMI- 1640. After reaching the confluency of about 80%, the cells were infected with either rPICV-H3 or rPICV-GFP at multiplicity of infection (MOI) of 1. After 1h adsorption in a 37°C, 5% CO2 incubator the virus inoculum was removed, and fresh medium was added to the wells. At 48 hrs post infection, expression of H3 antigen was evaluated by indirect immunofluorescence assay (IFA) using an inhouse monoclonal antibody (Vmab5) specific to the H3 antigen. For IFA, the cells were fixed for 10 minutes with 350 µl cold solution of methanol/acetone (1:1 v/v). After methanol/acetone was removed, the cell monolayer was allowed to air dry completely. The cells were rehydrated with 350µl of PBS followed by an incubation with a in house purified monoclonal antibody (Vmab5) specific for H3 protein for 1 hr at room temperature. After three washes in PBS, the cells were incubated with Alexa flour 488 labeled donkey anti-mouse IgG antibody (Invitrogen, Cat. # A21202) for 1 hr at room temperature. The cell monolayer was washed three times with PBS and observed under an inverted fluorescence microscope. Cells that were infected with rPICV-GFP were directly visualized under a fluorescence microscope at 48 hrs post infection. All the images were taken at 20x magnification.

3. Animal study

A total of 16 IAV-S seronegative 3-week-old pigs were randomly assigned into 4 different groups (T01-T04) accordingly as mentioned later. Two pigs died at the beginning of the study due to unknown reasons and were eliminated from the study. After 2 weeks of acclimatization, group

T01 (n=3) was injected with PBS to serve as a non-vaccinated control. Group T02 (n=3) was inoculated intramuscularly (IM) with 1x106.0 PFU of rPICV-GFP diluted in 2 ml of DMEM. Group T03 (n=4) was inoculated IM with 1x106.0 PFU of rPICV-H3 in 2ml of DMEM. Group T04 (n=4) was inoculated IM with 2mL solution containing 100µg recombinant H3 protein emulsified in 20% (V/V) Emulsigen-DL 90 (Phibro Animal Health Corporation, Omaha, NE). The immunization was administered once on day 0 and boosted again on day 21 intramuscularly. Group T01 and T02 were commingled in the same room. Nasal swabs were taken from all pigs on day -10 and -2, and 42 days post immunization to check for the presence of IAV-S RNA by commercial qPCR. Whole blood samples with anticoagulant EDTA were collected from all pigs at different time points (-10, 0, 14, 21, 28, 35 and 42-days post immunization) for the isolation of plasma samples to measure humoral immune response. On day 42 all the animals were challenged with 2mL inoculum containing 105.0 $TCID_{50}$/ml wild type H3N2 IAV-S strain by intra-tracheal route. Nasal swabs were collected from all the animals everyday post challenge infection to evaluate influenza virus shedding by qPCR. At day 5 post challenge (DPC) all the pigs were humanely euthanized. Bronchioalveolar lavage fluid (BALF) samples were collected in cold PBS to measure viral titers. Samples of lung and trachea were collected in 10% neutral buffered formalin for pathological evaluation by a board-certified pathologist blinded to the treatment groups. The experiment was approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee under the protocol number 1789.

4. ELISA

Enzyme-linked immunosorbent assay was performed with the plasma samples collected at different time points before the challenge to detect the presence of antibody against antigen delivered by the vector. Purified H3 protein was diluted to a concentration of 2µg/ml in PBS (pH-

7.4) and100µl was added to coat each well of a 96 well Immulon 2HB flat Bottom plate (Immunochemistry technologies, Cat. # 4073273) and incubated overnight at 4°C. After that, the coating solution was removed, and the wells were blocked for 2 hrs with 250µl/well of blocking buffer (10% skim milk in PBS with 1% Tween-20). The plasma samples were serially diluted two times in dilution buffer (5% skim milk in PBS with 1% Tween-20) and 100µl of each sample were added to the wells, followed by 1hr incubation at room temperature. After five washes with 300µl wash buffer per well (PBS with 1 % tween-20), goat anti-pig IgG antibody labelled with HRP (0.5mg/ml- KPL #5220-0363) was diluted 5,000 times in dilution buffer and 100µl was added to each well, followed by 30 mins incubation at room temperature. After five washes with the wash buffer, 100µl of ABTS substrate (Sera Care, KPL #5120-0042) was added to each well and the plate was incubated at room temperature for 5-25 mins. The reaction was stopped by adding 100µl of 1% Sodium dodecyl sulfate diluted in distilled water. Absorbance was read at 405nm. A similar ELISA procedure was used to measure antibody against GFP. In this case, ELISA plate was coated with 100 µl of 5µg/ml GFP protein (Sino Biologics, Cat # 13105-S07E).

5. Virus neutralization assay

Virus neutralization assay was performed in MDCK cells. Cells were seeded into 96 well plates at the density of 1.5x103.0 cells per well and cultured for 48 hrs. Plasma samples were incubated at 56°C for 30 mins to inactivate complement. Samples were diluted 2-fold serially in 50µl virus inoculation medium as mentioned in cells and reagents. An equal volume (50µl) containing 100 $TCID_{50}$ of IAV-S H3N2 TX98 was added to each well. After 1hr incubation at 37°C, the total mixture (100 µl/well) was transferred to another 96-well plate containing confluent MDCK cells which had been seeded 48h earlier. The plate was further incubated for 48 hrs at 37°C in a humidified atmosphere containing 5% CO2. After incubation, presence of virus infection was

determined by using an IFA assay as described previously in section 2. Neutralization titers were expressed as the reciprocal of the highest dilution that completely inhibited virus replication.

6. Hemagglutination inhibition assay

The plasma samples were first treated with receptor destroying enzyme (RDE) over night at 37°C, followed by an incubation at 56°C for 1hr to inactivate the RDE activity. The treated samples were then incubated with 50% turkey red blood cells (RBC, Lampire Biological Laboratories, Cat # 7209403) to remove non-specific binding proteins. Treated samples were diluted 2-fold serially in 25µl of PBS in V-bottom 96-well plates. Twenty- five µl of virus inoculum containing 8 HA units was added to all the wells except first column followed by 30 mins incubation at room temperature. After that 50µl of 0.5% RBC was added to all the wells, followed by 30 min incubation at room temperature. Hemagglutination inhibition (HI) titers were determined as the reciprocal of the highest sample dilution that completely inhibit hemagglutination.

7. Quantification of viral load

Total RNA was extracted from nasal swab and BALF samples by using the RNA extraction kit (Zymo research, Cat. # 1154G46), according to the manufacturer's protocol. The viral load was quantified using real-time reverse transcription PCR kit (RT-PCR, Life Technologies, VetMax-Gold SIV Detection Kit, Cat. #4415200) following the manufacturer's instruction in QuantStudio3 (Appliedbiosystems). A set of synthesized RNA template with known copy numbers was used to establish a standard curve from which the genomic copy number of the samples were extrapolated.

Virus titration assay was used to measure viral infectious units in BALF. MDCK cells were seeded in 96-well plates at density of 1.5x103.0 cells per well and cultured for 48 hrs. BALF samples were diluted 10-fold serially in the virus inoculation medium. After that, 100 µl of each

dilution were inoculated into each well of the 96-well plates containing MDCK cells cultured 48 hrs prior. After 1 hr adsorption, the BALF samples were removed, and the wells were replenished with 200µl of virus inoculation medium. After 48 hrs of incubation at 37°C, IFA in section 2 was performed to visualize the infected cells. Virus titers were calculated using a method described by Reed and Muench [22] and expressed as Tissue Culture Infectious Dose 50 ($TCID_{50}$) per milliliter.

8. Pathologic evaluation

Gross macroscopic lung lesions were scored at the time of necropsy by a board-certified pathologist who was blinded to the experimental design. The score was assigned based on the average percentage of each lung lobe affected by pneumonia with respect to the total lung volume [23, 24].

Sections of lung lobes and trachea were H&E (hematoxylin and eosin) stained and evaluated for microscopic lesion. Each lung section was evaluated and scored using 6 different parameters including interstitial pneumonia (0-4), peribronchiolar lymphocytic cuffing (0- 4), alveolar and interlobular edema (0-4), epithelial exocytosis (1-3), bronchi and bronchiolar epithelial changes like loss of cilia, necrosis, and proliferation (0-4), and bronchitis and bronchiolitis (0-4) with a score of 0 representing no change whereas a score of 4 representing severely affected [44, 93]. A composite score (sum of 6 individual scores) was reported for each section.

To look for the presence of viral RNA in the tissue samples, RNA in-situ hybridization was performed on formalin-fixed, paraffin-embedded (FFPE) lung and trachea sections as described previously [25]. Briefly, 4µm tissue section on the slides were baked for 1 h at 60 °C, then immersed in xylene for deparaffinizing, followed by dehydration in 100 % ethanol. Afterwards, tissue sections were treated with RNAscope® Hydrogen peroxide reagent (H2O2, ACD, Cat. #

322330) for 10 min at room temperature and washed twice with distilled water. The slides were immersed in boiling RNAscope® target retrieval reagent (ACD, Cat. # 322000) for 15 min and washed in distilled water followed by 100% ethanol. A hydrophobic barrier was drawn around the tissue sections. Protease plus reagent (ACD, Cat. # 322330) was added on to each tissue sections and incubated for 30 min at 40°C in a HybEZ hybridization oven. The sections were then incubated with a specific probe for IAV-S NP (V-InfluenzaA-H3N2-NP) for 2 h at 40 °C in a HybEZ hybridization oven. Signal amplification and detection reagents were applied sequentially using RNAscope® 2.5 HD Detection Reagent-Brown (ACD, Cat. # 322310) and incubated in AMP 1, AMP 2, AMP 3, AMP 4, AMP 5, and AMP 6 reagents, for 30, 15, 30, 15, 30, 15 min, respectively with two times wash in wash buffer reagent (ACD, Cat. # 310091) between each amplification step. Hybridization was revealed by using diaminobenzidine (DAB), followed by Gill´s hematoxylin counterstaining. The Ss-PPIB (Sus Scrofa Peptidylprolyl Isomerase B (cyclophilin B) probe the dapB probe were used as the positive and negative control, respectively. The frequency of IAV-S infected cells was scored by a board-certified pathologist using the score system ranging from 0 to 4 with 0 representing no signal and 4 represent the highest possible signal. The hybridization signals were evaluated in two different areas: airway epithelium and pulmonary parenchyma and a composite score (sum of scores from these two areas) was reported.

9. Statistical analysis

All the statistical analysis were carried out using the GraphPad Prism 9.3. Log2 titer for the H3 ELISA was analyzed using mixed-effects analysis multiple comparison. qPCR for viral RNA copy number in BALF, viral titer in BALF, macroscopic lung lesion score, in situ composite score for lungs and trachea and microscopic lesion score were analyzed by ordinary one-way analysis of variance (ANOVA). OD values for GFP ELISA, log10 viral titer in nasal swab, log2

virus neutralization titer, and HAI log2 titer were analyzed using 2way ANOVA. All the groups were compared with each other at different time points. p value <0.05 were considered statistically significant. Asterisk was used to denote statistically significant: * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

RESULTS

1. rPICV vector expressed high levels of H3 antigen in pig cells

We first evaluated the expression of the gene of interest (either H3 antigen or GFP) in different pig cells including PK-15 and PAM. BHK-21 which is routinely used to propagate rPICV was included to the study for comparative purposes. The cells were infected with rPICV-H3 or rPICV-GFP at MOI of 1. At 48hrs post-infection, GFP expression was directly visualized using an inverted fluorescence microscope (Nikon, Eclipse TS2R). As shown in Figure 2.2, GFP expression was observed in all three types of cells: BHK-21, PK-15, and PAM. The expression of H3 antigen was evaluated by an IFA using a monoclonal antibody specific to H3 protein. Again, IFA positive signal was observed in all three cell types tested. Collectively, the results indicate that rPICV can deliver genes of interest in pig cells.

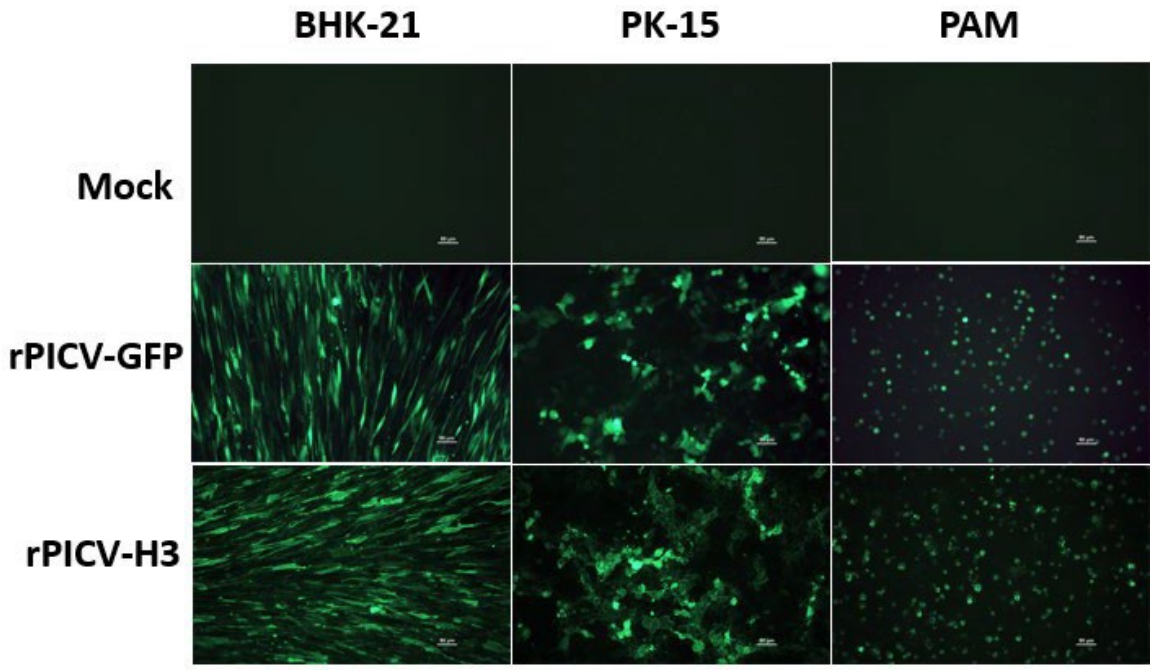


Figure 2.2. Antigen expression in swine cells.

Expression of GFP and H3 protein in the three different cell types BHK-21, PK-15, and PAM infected by rPICV-GFP and rPICV-H3 respectively.

2. Antibody responses against the viral NP protein

We first measured antibody responses against the viral NP protein using a commercial ELISA kit that is routinely used for IAV-S serodiagnosis. All pigs tested negative for NP antibody before vaccination (day -10) and continued to be negative until day 42 post vaccination (Figure 2.3). The results clearly demonstrate that the pigs used in this study were not exposed to IAV-S throughout the study and that the experimental vaccines did not induce antibody against NP protein.

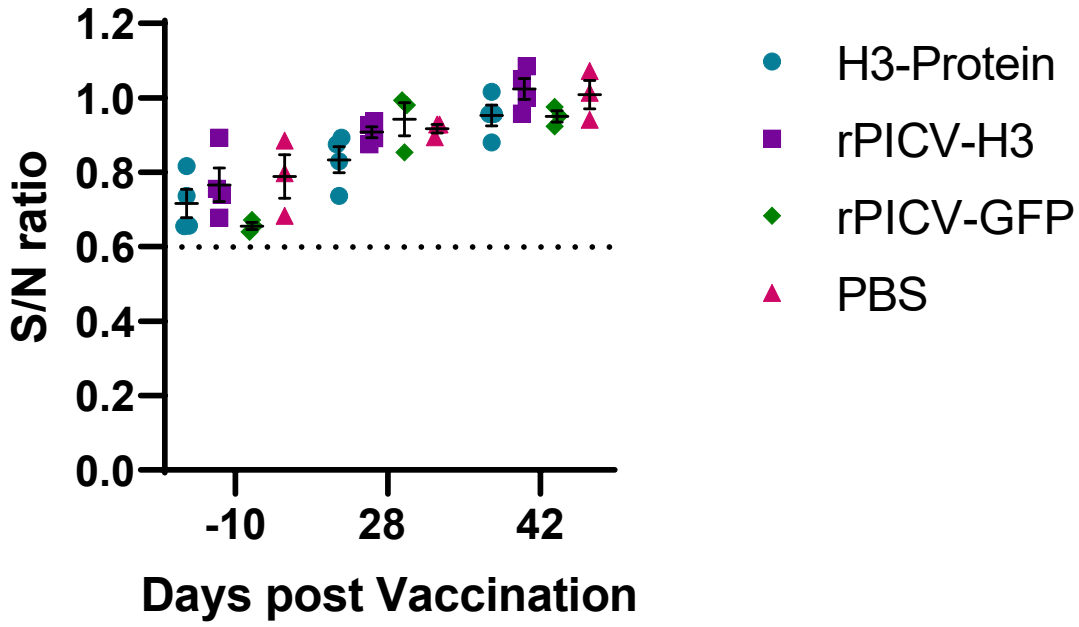


Figure 2.3. Antibody responses against the viral NP protein.

Serum samples collected before and after vaccination were used for a commercial inhibition ELISA that detects antibody against IAV-S NP protein. Data are express as the sample to negative ration (S/N ratio). The horizontal dotted line at S/N 0.6 indicates the assay cut-off. Samples with S/N above this cutoff are consider negative. T01, T02, T03 and T04 refers to group immunized with Mock, rPICV-GFP, rPICV-H3 and purified H3 protein respectively.

3. Antibody responses against GFP

To determine if rPICV was shedding and spreading from infected pigs, groups T01 (injected with PBS) and T02 (inoculated with rPICV-GFP) were commingled in the same room throughout the course of this study. Antibodies against GFP were measured in plasma samples collected at multiple time points before and after inoculation using an indirect ELISA. As shown in Figure 2.4, pigs inoculated with rPICV-GFP (T02) had GFP-specific antibodies whereas pigs inoculated with PBS did not. The results suggest that pigs inoculated with rPICV did not transmit the virus to the contact pigs.

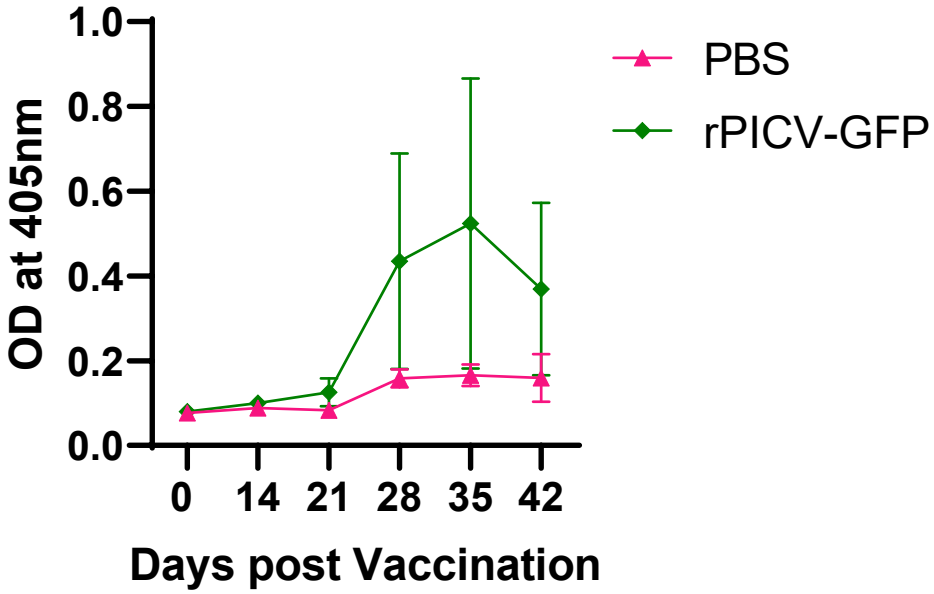


Figure 2.4. Antibody response against GFP.

Plasma samples collected at different time points after vaccination were subjected to an indirect ELISA that detect antibodies against GFP. Data expressed as mean and standard error of mean of optical density (OD) at 405nm.

4. Antibody against H3 protein

Antibodies specific to H3 antigen were measured by an indirect ELISA. H3-specific antibodies were not detected in pigs in T01 and T02 until day 42, the day of challenge infection with a live H3N2 virus. In contrast, pigs in T03 and T04 groups exhibited high titers of H3-specific antibodies (Figure 2.5). H3-specific antibodies could be detected in T04 group (immunized with recombinant H3 protein with adjuvant) at day 14 after the first dose of immunization whereas these antibodies were not detected in T03 group (immunized with rPICV-H3) until day 28, corresponding to day 7 after the second immunization. However, no significant difference in the antibody titer between T03 and T04 was observed one week after the second immunization. The results indicate that immunization of pigs with recombinant H3 protein together with adjuvant elicited faster antibody responses than immunization with the rPICV-H3 viral vector.

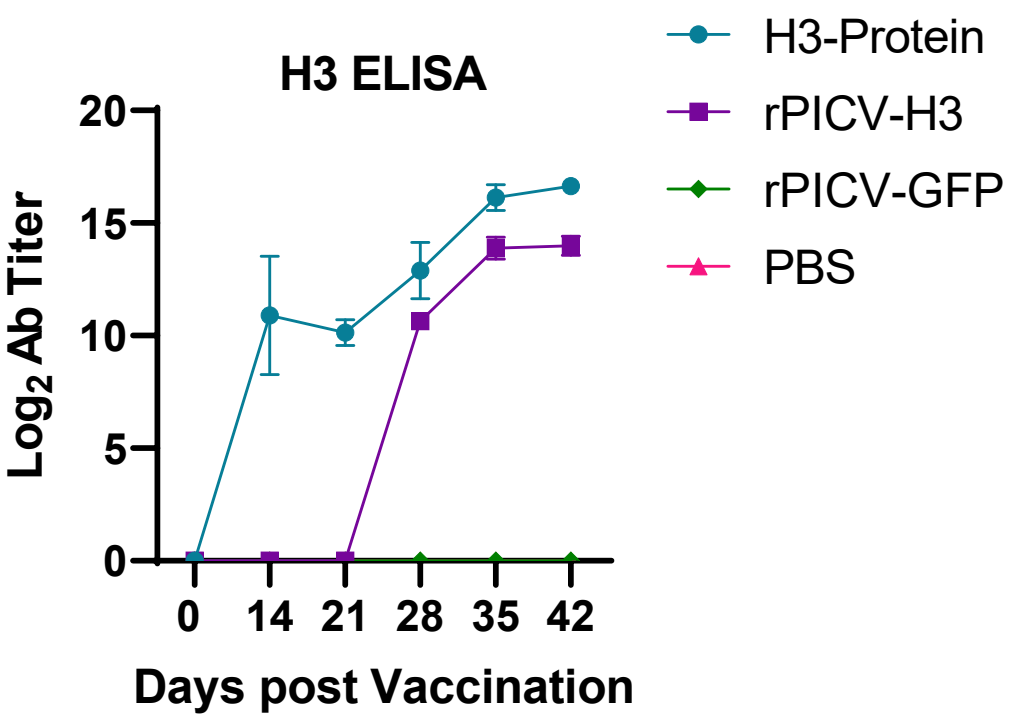
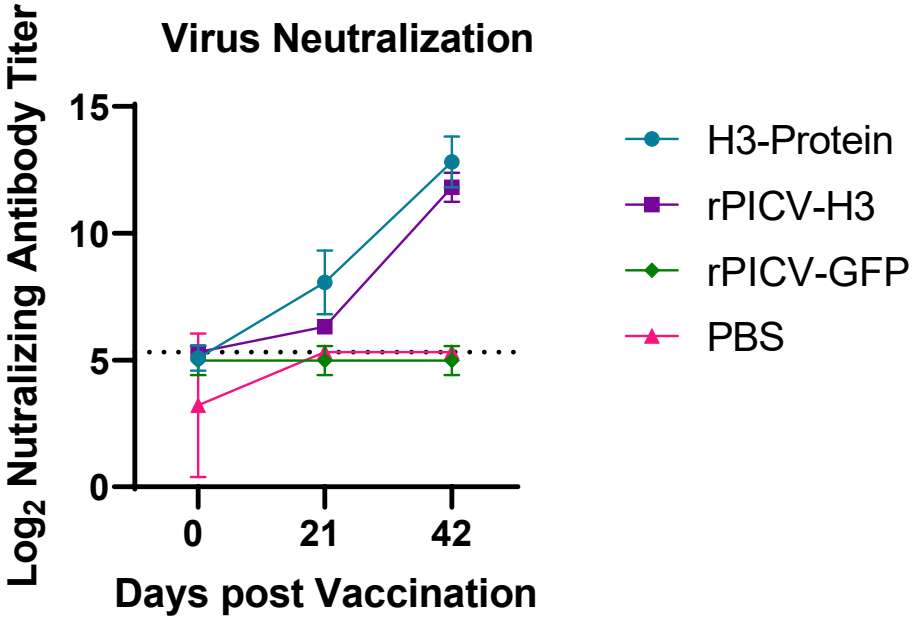


Figure 2.5. H3-specific antibody responses.

Plasma samples collected at different time points were assayed for the presence of H3-specific antibodies by an indirect ELISA. To determine the antibody titers, a cutoff equivalent to mean plus 5 standard deviation OD values of pigs in T01 and T02 group was calculated. Samples with OD values greater than the cutoff were consider positive. The end-point antibody titers are expressed as the $\log_2$ of the reciprocal of the highest serum dilution that tested positive by the ELISA. Data expressed as mean and standard error of mean of optical density (OD) at 405nm.

5. Virus neutralization and hemagglutination inhibition antibody responses

Next, virus neutralization (VN) and hemagglutination inhibition (HI) antibody titers were evaluated. Background levels of VN and HI antibody titers (approximately 1:40) were observed in all pigs before immunization. VN and HI antibody titers did not increase in T01 and T02 group whereas these antibody titers significantly increased in T03 and T04 at 21 DPV and increased even further at 42 DPV. No significant difference in VN and HI titers between T03 and T04 groups were observed (Figure 2.6). The results indicate that both rPICV-H3 and recombinant H3 vaccine were able to elicit functional antibodies that block IAV-S infection.



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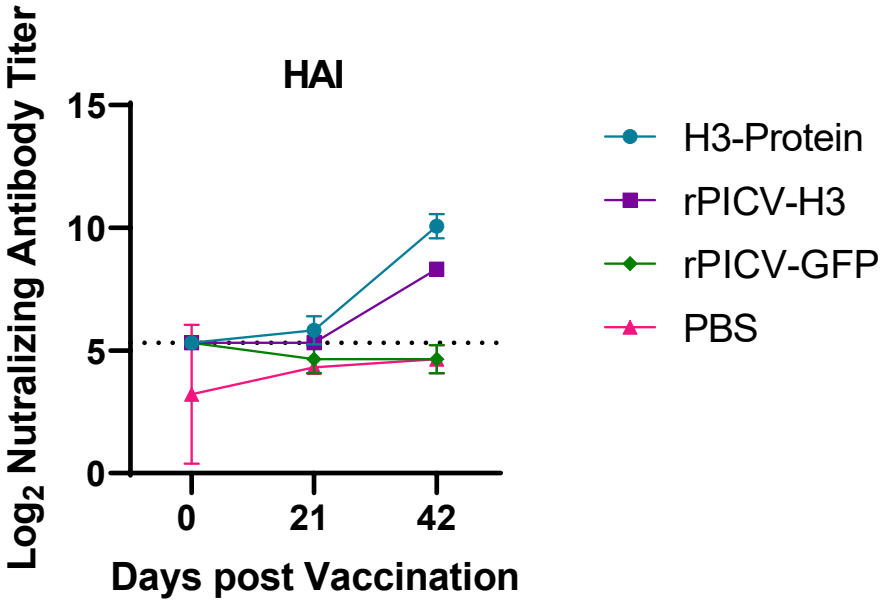


B

Figure 2.6. Virus neutralizing (VN) and hemagglutination inhibition (HI) antibody responses.

(A) Plasma samples from 0, 21 and 42 days post vaccination (DPV) were used to determine the virus neutralization titers against H3N2 TX98 in the MDCK cells. (B) Plasma samples from 0, 21 and 42 DPV were used to determine the hemagglutination inhibition titer against H3N2 virus. Horizontal dotted lines indicate the cutoff of the assays at 5.32 Log_2 neutralizing antibody titer equivalent of 1:40 times sample dilution. Data expressed as mean and standard error of mean of neutralizing antibody titer.

6. Detection of IAV-S RNA in nasal swab samples

All animals were challenged by an intra-tracheal inoculation with the H3N2 TX98 strain at 42 DPV. Nasal swabs were collected daily from all animals to measure the IAV-S shedding. Viral RNA was detected from all pigs in T01 and T02 groups starting from day 1 post-challenge. On the other hand, viral RNA was not detected in any of the pigs from T04 group at any time-points post challenge (Figure 2.7). For T03 group, two pigs did not have any detectable levels of viral RNA at any time points post challenge. The other two pigs had detectable viral RNA at only one time on day 1 and day 4 post challenge.

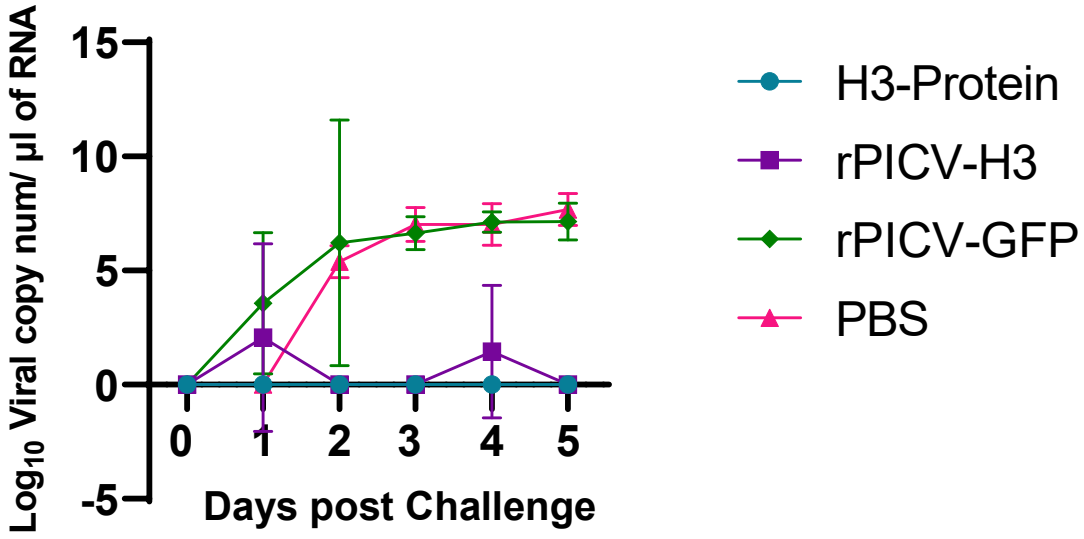


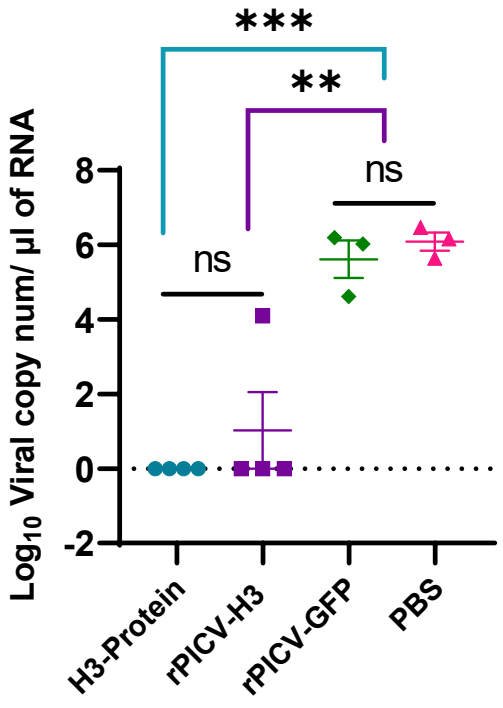
Figure 2.7. Detection of IAV-S NP RNA in nasal swab samples

Nasal swabs collected daily after challenge infection were subjected to a quantitative real-time PCR to determine the amount of viral RNA in the samples. Data are expressed as mean and standard error of mean of the viral RNA copy number per µl of RNA sample from all animals of one group.

7. Detection of viral RNA and infectious virus in BALF

All pigs were humanely sacrificed at 5 DPC and BALF was collected to measure viral RNA by using a real-time PCR. High levels of viral RNA were detected in BALF from all pigs in T01 and T02 groups (Figure 2.8A). In contrast, viral RNA was not detected in BALF from any pigs in T04 group. Whereas one of the pigs in T03 group had IAV-S NP RNA. This pig also had viral RNA in its nasal swab collected at 4 DPC.

To determine viral infectivity, samples of BALF were titrated in MDCK cells. All BALF samples collected from pigs in T01 and T02 groups had infectious virus with the titer ranging from 10^4-$10^{5.75}$ $TCID_{50}$/ml (figure 2.8 B). On the other hand, infectious virus was not detected in BALF of any pigs in T03 and T04 group. Even though BALF from one pig in T03 group exhibited detectable level of viral RNA, this sample was not infectious when inoculated onto MDCK.



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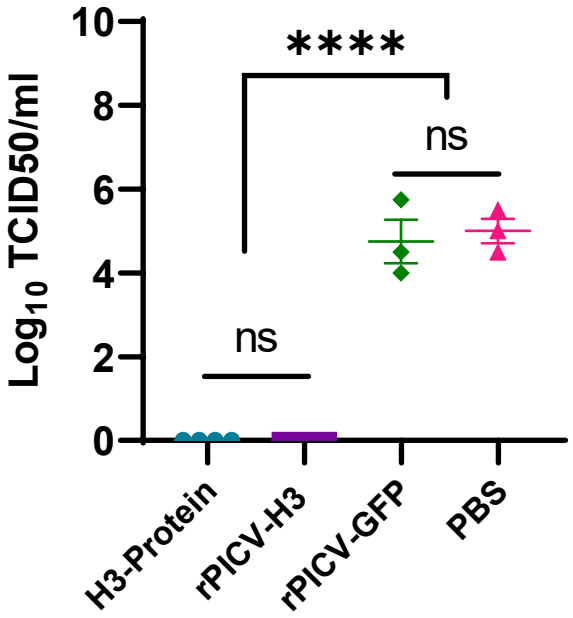


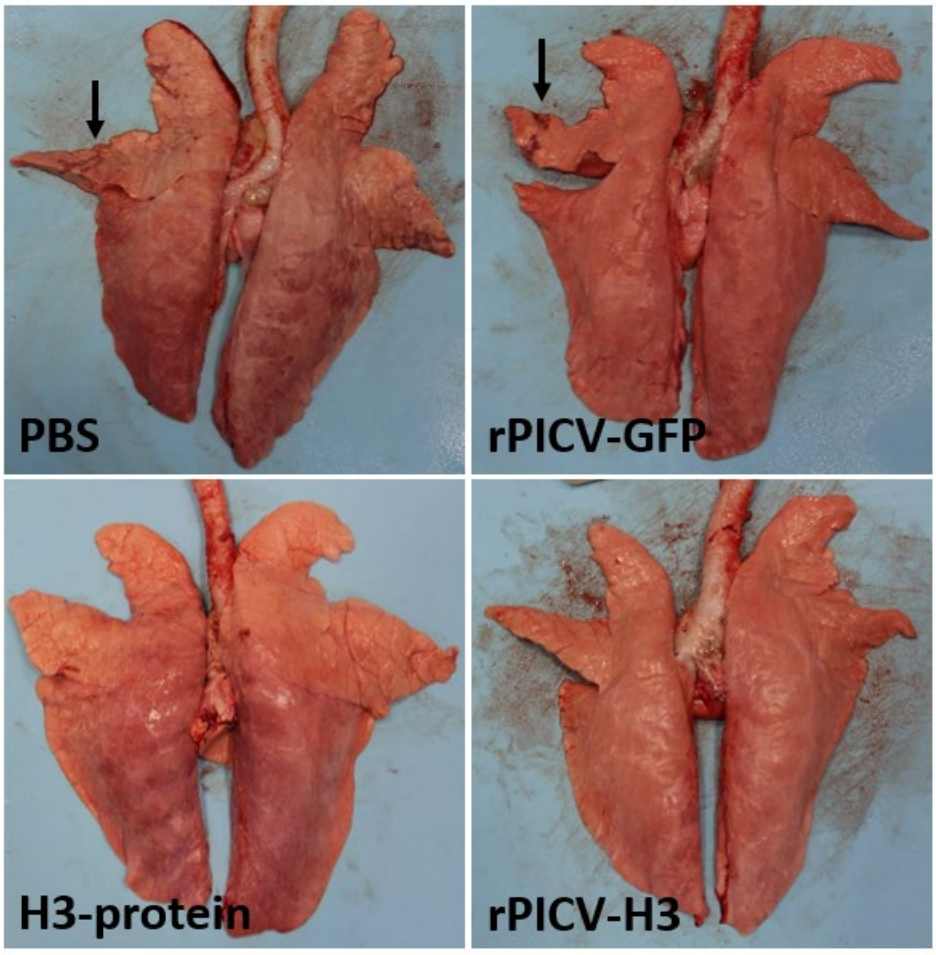
B

Figure 2.8. Detection of viral RNA and infectious virus in BALF.

(A) RNA was extracted from BALF and used for quantitative real-time PCR. Data are expressed as log_{10} viral genome copy number per µl of RNA sample. (B) Infectious virus in BALF were titrated in MDCK cells. Data are expressed as log_{10} $TCID_{50}$ per mL of sample and Error bars indicate the standard error of mean value. ns-No significance, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

8. Lung pathology

Typical macroscopic lung lesions were observed in pigs T01 and T02 groups with the mean lung consolidation ranging from 2% to 4% (Figure 2.9). The lesions were more profound on the left cardiac lung lobe. On the other hand, lung consolidation was not observed in pigs from T03 and T04 groups.



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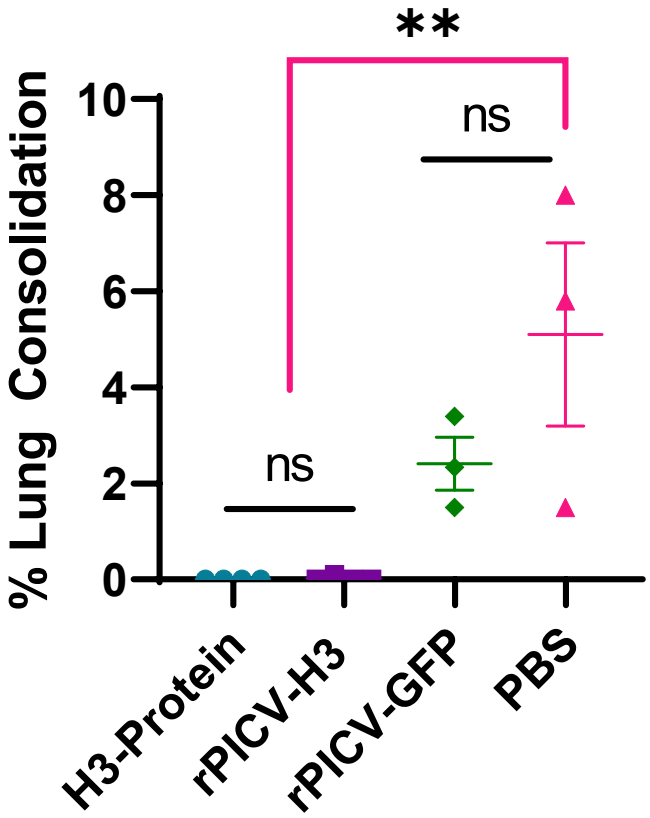
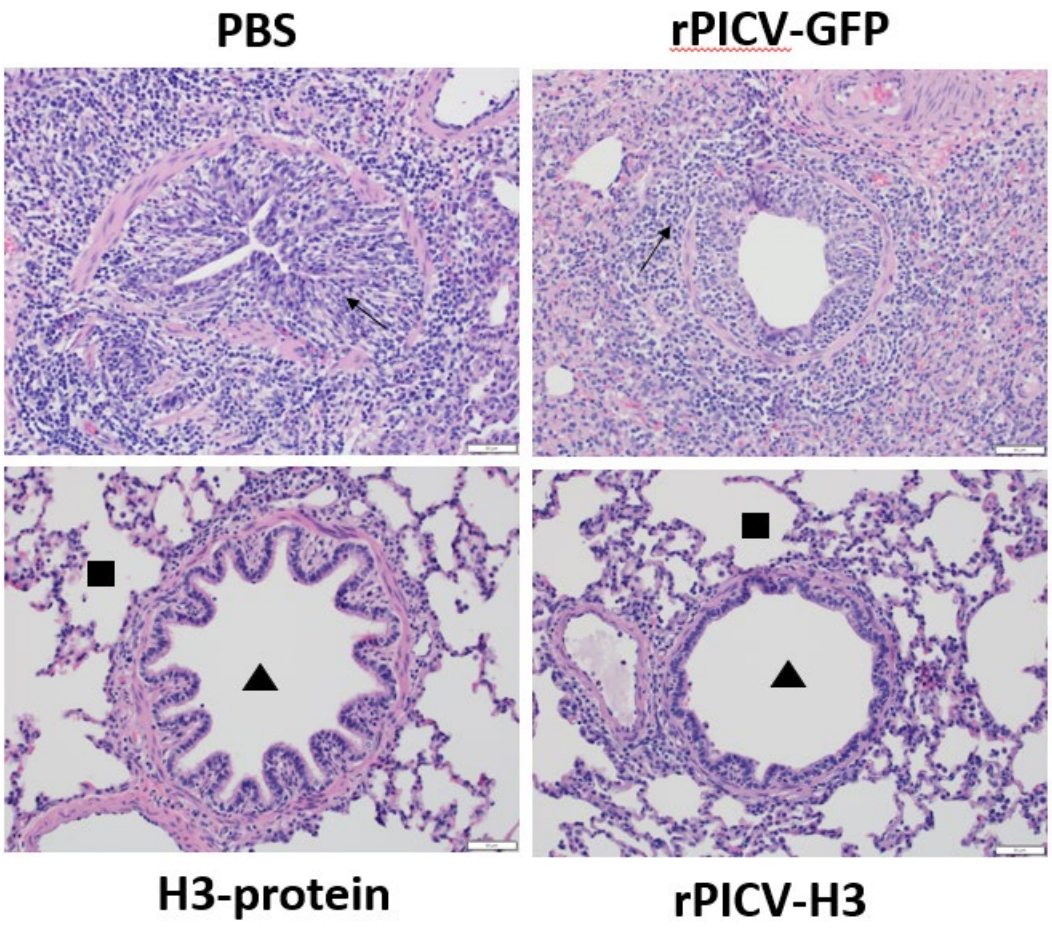


B

Figure 2.9. Macroscopic lung lesion scores.

(A) representative images for presence of lung lesion indicated by the black arrows pointing at red depression in the lung of pigs from different groups. (B) percentage lung consolidation quantifying the scoring of macroscopic lesion in lungs for all the groups. Data expressed as mean and standard error of mean % lung consolidation for all the animals in each group. ns-No significance, ** $p \leq 0.01$

Microscopic lung lesion scores followed a similar trend as the macroscopic scores. Pigs in T01 and T02 groups exhibited significantly high microscopic lesions characterized by necrotizing bronchiolitis with peribronchiolar lymphocytic cuffing and interstitial pneumonia. The mean composite microscopic scores for pigs in T01 and T02 groups varied from 5 to 7 (Figure 2.10). On the other hand, no microscopic lesions were observed in T03 and T04 groups.



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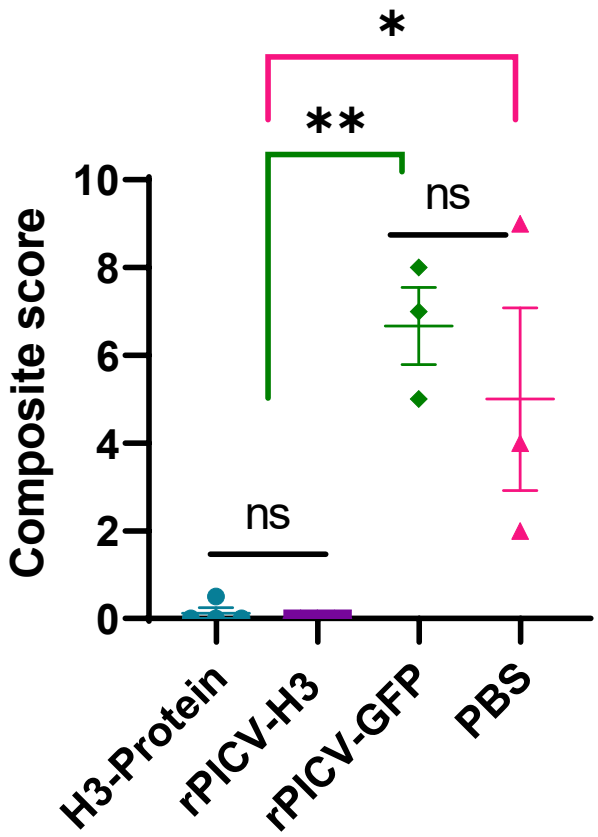


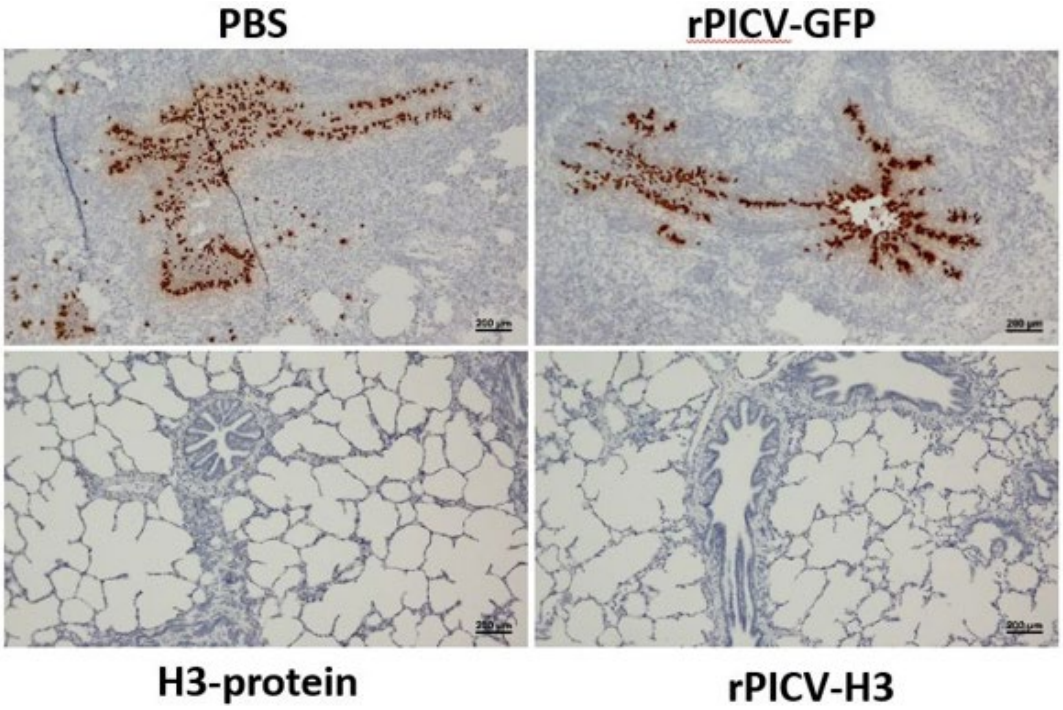
B

Figure 2.10. (A) representative images of H&E-stained lung tissue samples (B) composite score comparing microscopic lesion scores between all the groups.

Arrows pointing at cellular infiltration in the bronchioalveolar space. Squares and triangles pointing at clear alveolar and bronchiolar space respectively. Data expressed as mean and standard error of mean microscopic lesion composite scores for all the animals in each group. ns- No significance, * $p \leq 0.05$, ** $p \leq 0.01$

9. Detection of virus infected cells in lung and trachea

RNA in situ hybridization was used to detect virus infected cells from lung and trachea sections. As shown in Figure 2.11, large number of virus infected cells were observed in both lung and tracheal sections of pigs in T01 and T02 groups. In contrast, no virus infected cells were observed in lung and trachea section of pigs in T03 and T03 groups



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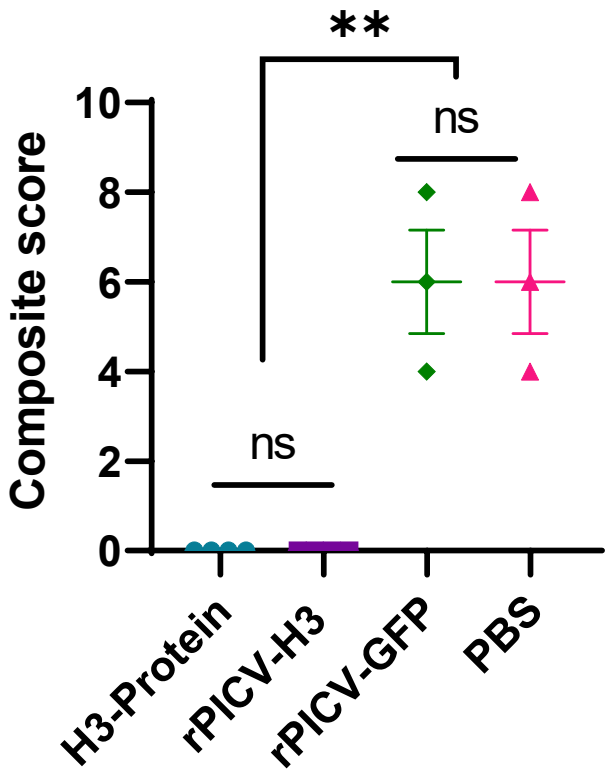
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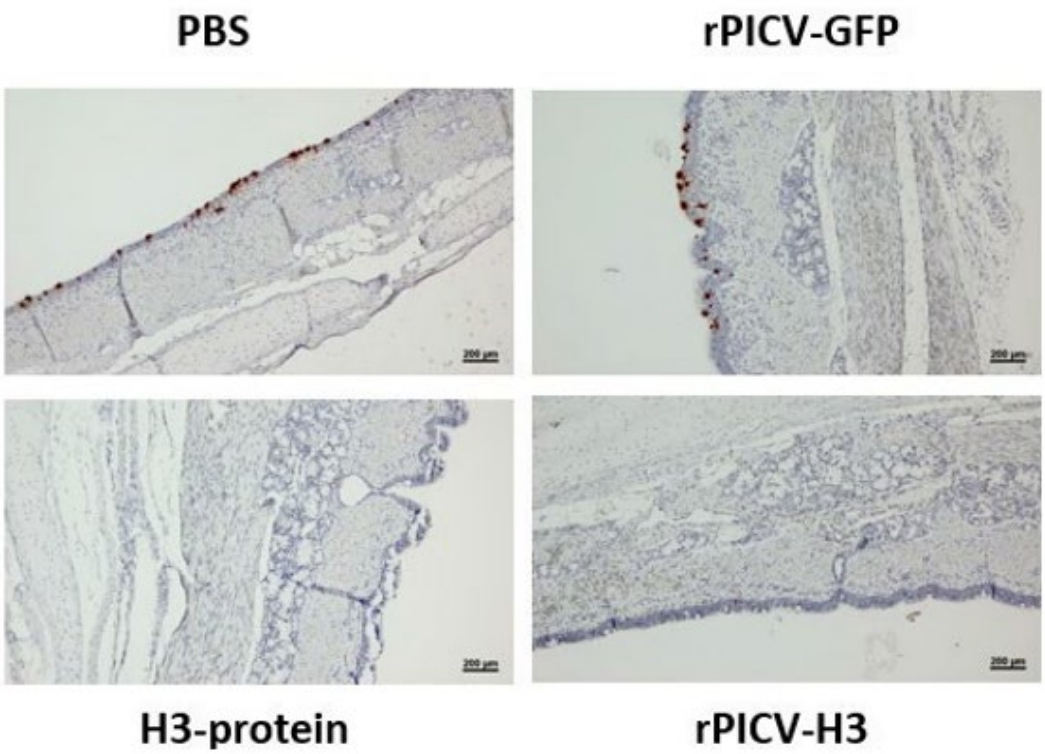
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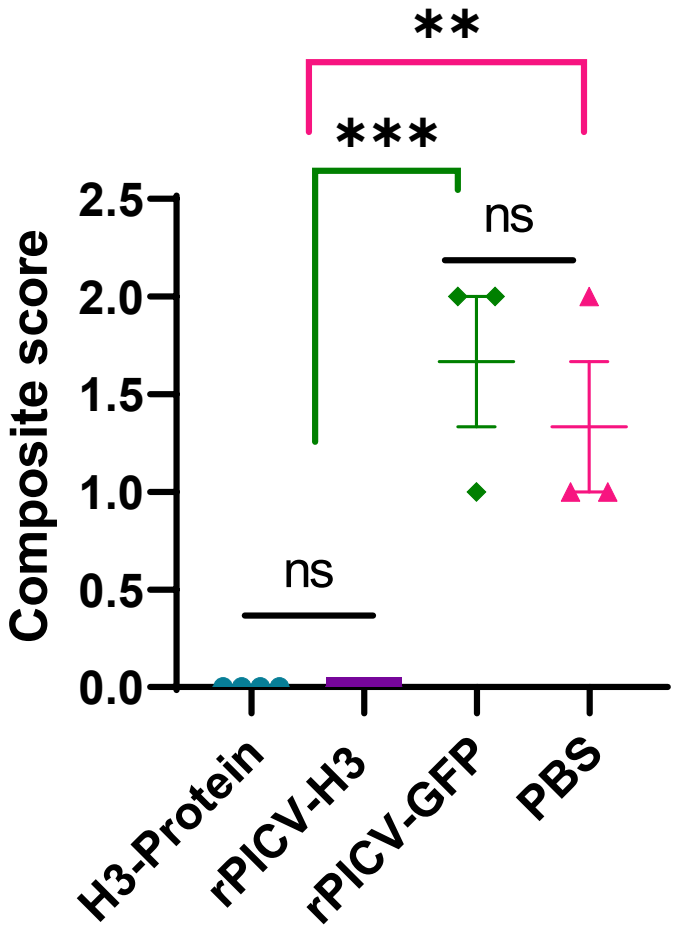


D

Figure 2.11. In situ for IAV-S RNA in the tissue

Lung (A) and trachea (C) tissue section showing presence of IAV-NP RNA. Quantitative comparison of presence of viral RNA in the lung(B) and trachea (D) samples. Data expressed as mean and standard error of mean composite scores for all the animals in each group. ns-No significance, ** $p \leq 0.01$, *** $p < 0.001$

DISCUSSION

Swine influenza virus, first isolated from pigs in 1931 [19], were found to have negative sense segmented RNA genome. This inherent property of the flu virus genome allows it to frequently mutate and generate new variants either by exchanging RNA segments between different antigenic strain or due to accumulation of point mutations because of error prone replication. Most efficient way of protecting the pigs is by preventing the spread of the virus by vaccinating the animals against influenza virus [34].

Majority of the licensed vaccines for pigs are based on the whole inactivated viruses, which are safe and provides robust protection against the homologous infection but fails to show any efficacy when a heterologous strain of virus infects the animals. In the presence of maternally derived antibodies, vaccination of piglets with WIV enhances the respiratory disease pathology rather than protecting them [45]. The live attenuated or modified live vaccine for influenza A virus protects the pigs from homologous infection and shows some level of efficacy against heterologous infection as well. But it must be noted that the LAIV is a replicating virus and the fact that it may reassort with the wild type infectious IAV co-circulating in the field and revert to being infectious cannot be eliminated [47]. The vaccines based on RNA particle (Sequivity, Merck) technology is a good candidate for frequently updating the vaccine with HA genes from current circulating strain from a locality. But the challenge with the RNA particle technology is the requirement of large amount of recombinant RNA expression. Expressing large amount RNA can be difficult, expensive and time consuming, limiting the availability of the vaccine. The poor stability of the RNA particle is also another issue [94].

Using a recombinant virus that can infect the porcine cells and deliver the HA antigen into the system without causing any disease can be a useful alternative in influenza virus vaccine

development. Recombinant Pichinde virus is one such viral vector that can infect the porcine cells and express antigen very well, as shown by PK-15 and primary PAM cells infected with rPICV-GFP and rPICV-H3. One of the concerns associated with live viruses is that they can replicate and spread from one animal to another [95]. The PBS group pigs even after comingling with rPICV-GFP infected pigs in the same room for over 42 days, did not have any anti-GFP antibodies in the plasma sample as detected by ELISA. If the GFP protein expressing pichinde virus was shedding, pigs from PBS group would have picked them up thereby express antibodies against the protein. This indicated that the pichinde virus does not shed or spread from spread from one animal to another.

Interestingly, for humoral immune response, while the H3-protein immunized pigs expressed high titer of anti-HA antibodies after first vaccination, the rPICV-H3 immunized pigs expressed significant levels of anti-H3 antibodies only after the second immunization. On determining the functional response of these Anti-HA antibodies by virus neutralization and hemagglutination inhibition assay it was observed that even though the H3-protein group had presence of high level of antibodies against the HA protein starting from the first dose itself, significant levels of neutralizing antibody titer was found only after the booster immunization, just like that of the rPICV-H3 immunized pigs. This can be attributed to the fact that the antibodies expressed in H3-protein injected animals was not specific to the neutralizing epitope of the H3 protein and may be binding to some other region of the full length H3 protein, that was used for performing ELISA. This indicated that the early antibody response seen in the ELISA for H3 treated animals did not have a protective role. The high concentration of H3 protein used to vaccinate the pigs might have also contributed to the high levels of early antibodies.

When the vaccinated animals were challenged with homologous influenza virus strain, none of the pigs from H3 protein vaccinated group showed any viral RNA in the nasal swab or BAL fluid sample, whereas two of the four pigs from rPICV-H3 vaccinated group showed the presence of viral RNA at two different time points in the nasal swab sample and only one animal in the BAL fluid. When the same BAL fluid samples were used to detect the presence of infectious virus particle, it was observed that even though some of the rPICV-H3 vaccinated pigs had viral RNA they were not from infectious virus particle as none of the sample could infect or replicate in the MDCK cells compared to which virus in the BALF sample from PBS and rPICV-GFP group replicated in the MDCK cells with high viral titer. Along with these results the absence of any macroscopic or microscopic lung lesion and viral RNA in tissue of the infected animals indicated that the vaccination of rPICV-H3 completely protected all the pigs just like the H3 protein immunization.

Overall, it was concluded that recombinant Pichinde virus can be a potential viral vector for the development of swine influenza vaccines to administer in the pigs, providing solid protection against the homologous swine influenza challenge. In future rPICV construct with HA from other circulating subtypes of swine influenza virus belonging to H1N1, H1N2 and H3N2 [20] can be used to vaccinate the pigs and determine if the animals are protected and whether equal level of protective immune response is elicited against each subtype to eventually develop a broadly protecting vaccine against Influenza A viruses of swine.

CHAPTER 3: OPTIMIZATION AND EVALUATION OF A METHOD FOR MONOCLONAL ANTIBODY GENERATION

ABSTRACT

A new method for generation of monoclonal antibody (mAb) was evaluated in mice model for optimizing the method to express mAb from pigs. Antigen specific memory B cells were sorted out and cultured in-vitro with feeder cells expressing CD40L and exogenous recombinant cytokines IL-2 and IL-21. Variable genes were isolated from the cultured B cell clones expressing antibodies against the antigen and cloned in expression vectors containing constant region of mouse IgG heavy chain and light chain separately. The vectors containing full length heavy and light chain of the antibody were transfected into HEK-293T cells. Finally, ELISA performed for NSP7 protein with the transfected cell culture supernatant detected the presence of functional anti-NSP7 antibodies, indicating that the method used in this study for monoclonal antibody generation worked. In future this same technique can be applied to express monoclonal antibodies for species of other origin like pigs.

Expressed by single B cell clones, monoclonal antibodies are a homogenous mix of immunoglobulins targeting a specific epitope of an antigen [96]. Each antibody consists of a pair of identical heavy and light chains. The light chain weighing ~22kDa [97], has an amino terminal and a carboxy terminal. The amino terminal half consists of the variable region (V_L) and carboxy terminal possess the constant region (C_L). There are two major types of light chain constant sequences- κ and λ [98]. In humans there are 60% of light chains that are κ and 40% that belong to λ. But unlike humans, in mouse only 5% of the light chains are of λ-type [99]. The heavy chain constant region is larger when compared to the light chain, consisting of three different constant regions CH1, CH2, and CH3 at the carboxy terminal and a variable region V_H at the amino

terminal. The two heavy and light chain dimers are held together by a flexible hinge region. The heavy chain sequences can be of five different types referred to as - μ, δ, γ, ε, and α isotype, each isotype giving rise to a specific class of antibodies- IgM, IgD, IgG, IgE and IgA respectively. The molecular weight of heavy chain is approximately 55kDa [97] with 20% higher for IgM and IgE molecule. While the sequence of the variable region varies from one antibody to another within the same organism, constant domain sequence remains the same for a particular species [100]. It is to be noted that both the heavy and light chains are encoded by two different mRNAs.

Monoclonal antibodies are of great importance as a diagnostic tool, where they can be used to detect the presence of a pathogen, toxin, drug, hormone or they can be used to detect a certain cell type or molecule to determine its function [101]. There are many mAbs that has been used as therapeutic interventions too [102, 103]. The first FDA approved murine mAb OKT3 mAb targets human CD3 receptor on T cell surface to prevent the graft rejection in individuals who undergo organ transplants [104]. A mouse chimeric antibody rituximab is used to treat non-Hodgkin B cell lymphoma [105, 106]. Rituximab binds to the CD20 receptor present on tumor B cells and elicits an immune response against the malignant cells to kill them [107]. Humanized mAb palivizumab targeted against respiratory syncytial virus is approved by the FDA for the treatment of the infected children [108]. Apart from being a diagnostic or therapeutic tool, recently the usage of monoclonal antibody to guide the designing of structure-based vaccine has seen significant advancement. There are different ways in which the antibody can guide vaccine development. They can be either conformation-based designing, or epitope-based designing [109]. Immunogenic antigens can take more than one conformation [110], antibodies can be used to identify which conformation induces a protective immune response [111]. Epitopes, the sites present on an antigen that can be detected by an antibody or supersites consisting of multiple overlapping epitopes that can be recognized by

broadly protective antibodies can help in development of multivariant vaccines. In case of Influenza, broadly recognizing antibodies induced by vaccination with diverse hemagglutinin were shown to be binding to the hemagglutinin stem region [112]. New vaccines are already being studied using these supersites for the development of multivalent vaccines against influenza virus [113, 114].

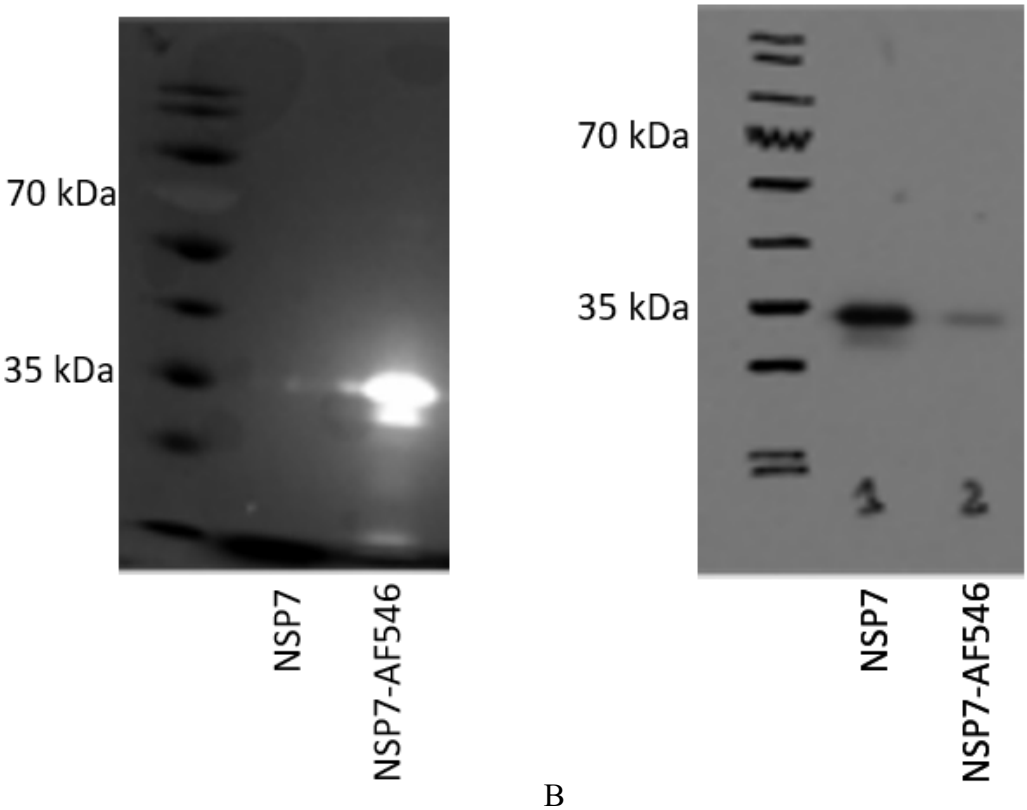
To generate optimum number of monoclonal antibodies for all these applications, they are expressed and purified in vitro. Monoclonal antibodies can be generated by different techniques. The classical method involves hybridoma technique [115]. In this method lymphocytes from the antigen stimulated animal are isolated from spleen and fused with the same species immortal myeloma cells. The fused cells are cultured in a hypoxanthine-aminopterin-thymidine (HAT) selection media. The aminopterin in the HAT media blocks the cells from synthesizing nucleotides by the de novo pathway. Hypoxanthine and deoxythymidine allows cells with functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT) to synthesize nucleotides via the salvage pathway [115]. Even though myeloma cell is immortal, they will dye in the HAT media, as it does not have HGPRT. B cells have short life span, so they eventually die too. Only the fused myeloma and B cells called hybridomas will be able to survive as combining immortality from myeloma cells and presence of HGPRT in B cells [116]. Successful hybridoma colonies are later screened for antigen specificity. The hybridoma cells can be cryopreserved for using any time in future [117]. But the challenge with hybridoma technique is that it requires a suitable fusion partner. For mouse, SP2/0 cells are the myeloma cell line that serve as the fusion partner [118]. But for pigs until now there is no known myeloma cell line that can be used to fuse with the B cells [119]. Fusion efficiency of mouse myeloma cells and swine B cells is very low and the few cells that successfully fuse to form hetero-hybridomas are highly unstable and lose antibody

expressing capacity after some time (as observed by us in our work), and thus makes it challenging to generate antibodies of swine origin using hybridoma technology. Another method to generate mAb depends on the separation of antigen specific single B cells from the immunized or infected donor's blood or splenocytes Antibody genes from the single B cells are isolated and PCR amplified to clone into expression vectors [120]. But isolation of RNA from the single B cell limits the amount of template available for PCR, making it difficult to amplify the antibody genes. To overcome this issue of limited template, another method of monoclonal antibody generation depends on growing the B cells for a short time span. Normally, in vivo, once the antigen displaying B cell encounters the antigen specific T cell, the T cell recognizes the MHC-II complexed antigenic peptides on the B cell's surface and secretes CD40L [121], costimulatory cytokines like IFN-γ, IL-4, IL-21 [122] and IL-2 [123]. The CD40L binds with the CD40 receptor present on the surface of the B cell, leading to proliferation and differentiation of the B cells. Interleukin-2 also aids the B cell proliferation and differentiation [124]. After the initial secretion of IgM, cytokines released by the T cells, especially interleukin-21 stimulates these plasma B cells expressing IgM to undergo Ig isotype switching [125], and produce IgG, in some cases IgA or IgE rather than IgM [126]. Following the same principal antigen specific B cells are cultured transiently in an in vitro setup along with feeder cells that express CD40L and co stimulatory cytokines IL-2 and IL-21, increasing the amount of template availability for PCR amplification [127, 128]. From this culture, B cell clones that are expressing antibodies targeting the immunogen of interest can be selected for isolation and cloning of antibody genes to generate monoclonal antibodies [129].

This study was performed for the optimization of B cell co-culture procedure as mentioned before [20, 129], that can be applied to generate monoclonal antibodies from pigs by eliminating

the need of any suitable fusion partner or low amount of antibody gene PCR templates. Due to ease of animal handling, antigen immunization, and sample processing we first evaluated the feasibility of the B cell co-culture method in mice. As purified recombinant PRRSV-NSP7 protein was already available with us, therefore the study was performed with this protein.

Firstly, NSP7 protein was labelled with a fluorophore to detect and separate antigen specific B cells in Fluorescent activated cell sorting (FACS). 1µg of NSP7 protein was incubated with Alexaflour-546 (AF546, Invitrogen, Cat#A20183) and conjugation was performed as per manufacturer's instructions. Labelling of NSP7 was confirmed by running SDS-PAGE for the labelled protein. Un-tagged NSP7 protein was run along with the tagged protein as a positive control. After the protein bands were transferred on to polyvinylidene difluoride (PVDF) membrane, it was observed under ChemDoc (Figure 3.1 A). While unlabeled NSP7 protein showed no bands in ChemDoc, the NSP7-AF546 showed bright band near 35kDa, same size as that of NSP7 protein. The same membrane was then probed with anti-His tagged primary antibody to detect the NSP7 protein with 6X His tag. After probing the membrane with HRP-labelled goat anti-mouse IgG (KPL, Cat#5220-0360) secondary antibody, membrane was incubated with HRP-substrate and bands were developed on a sheet of X ray film. It was observed that both untagged NSP7 and labelled NSP7-AF546 had distinct bands near 35kDa (Figure 3.1 B), implying that the image from ChemDoc where no bands for untagged NSP7 was observed was because of no AF546 labelling and not due to the absence of NSP7 protein.

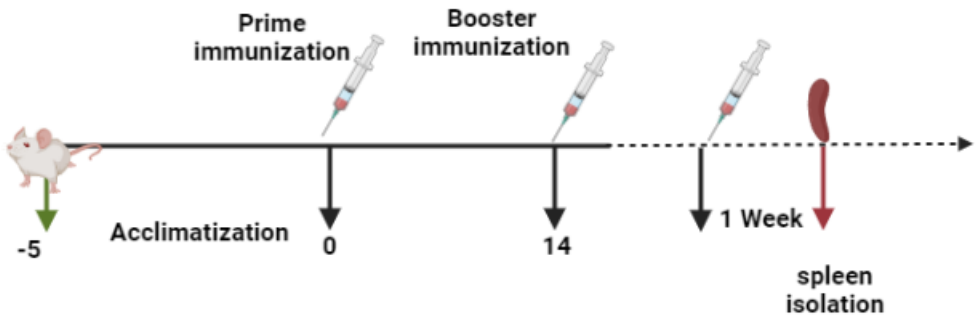


A B

Figure 3.1. Labelling of antigen with fluorophore.

(A) untagged NSP7 protein and AF546 tagged NSP7 protein bands after running SDS-PAGE as observed under ChemDoc. (B) His tag probed protein bands as observed on the X-ray film. (Where NSP7 is untagged protein and NSP7-AF546 is protein labelled with the fluorophore.

After the confirmation of successful antigen labelling, a group of 8 weeks old BALB/C mice were immunized with 10μg of NSP7 protein first emulsified in Freund's complete adjuvant, followed by boosting with same amount of protein emulsified in Freund's incomplete adjuvant (Figure 3.2 A). Serum samples were collected from the mice to perform ELISA against NSP7 protein in order to determine whether the mice were seroconverted or not (Figure 3.2 B). High level of absorbance detected in ELISA clearly indicated that the animals were well seroconverted and anti-NSP7 antibody was being expressed.



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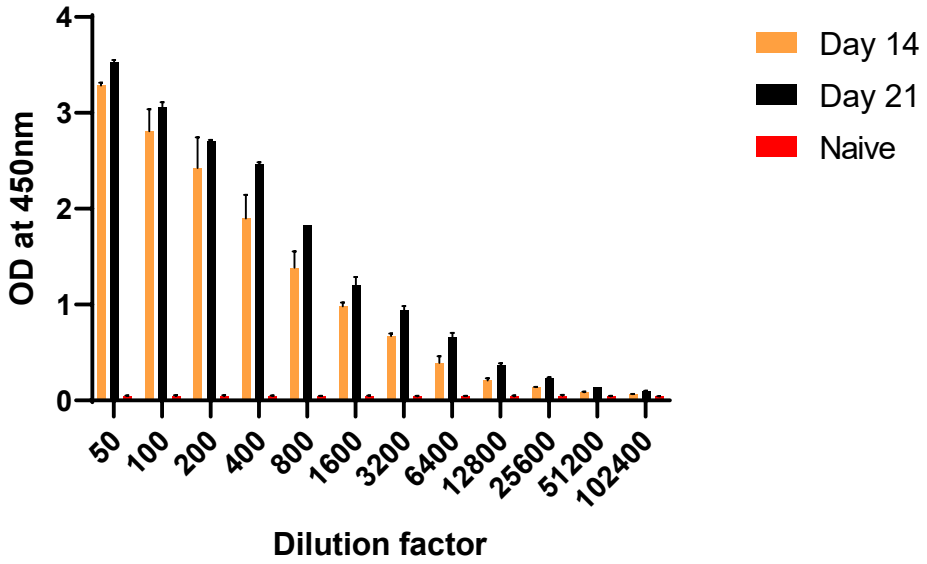


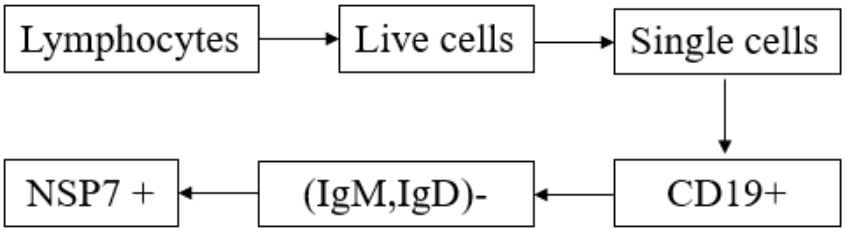
B

Figure 3.2. Immunization of mice.

(A) schedule of mice immunization and spleen collection. (B) graphic representing OD values from the ELISA against NSP7 using sera samples, showing seroconversion of mice. The data are represented as mean and standard error of mean for each OD value at 450nm.

One week after the booster immunization, splenocytes from an immunized mouse and another from a naïve mouse was isolated.

Both the samples were stained with four different cell surface markers: Zombie aqua (Biolegend, Cat#423101), anti-mouse CD19 APC (Biolegend, Cat#115512), IgM AF488 (Biolegend, Cat#406522) and IgD Brilliant violet 711 (Biolegend, Cat#405731) along with NSP7-AF546 all together and sorted by FACS instrument to obtain IgG expressing memory B cells. Zombie Aqua was used to separate the live cells from total lymphocytes. CD19, a pan B cell marker, was used to gate B cell population from live cells [130]. For the isolation of memory B cells, IgM and IgD double negative population was selected from CD19+ cells [131]. Finally, to detect the antigen specificity, IgM, IgD double negative (DN) cells were sorted for NSP7-AF546 stained cells (Figure 3.3 A).



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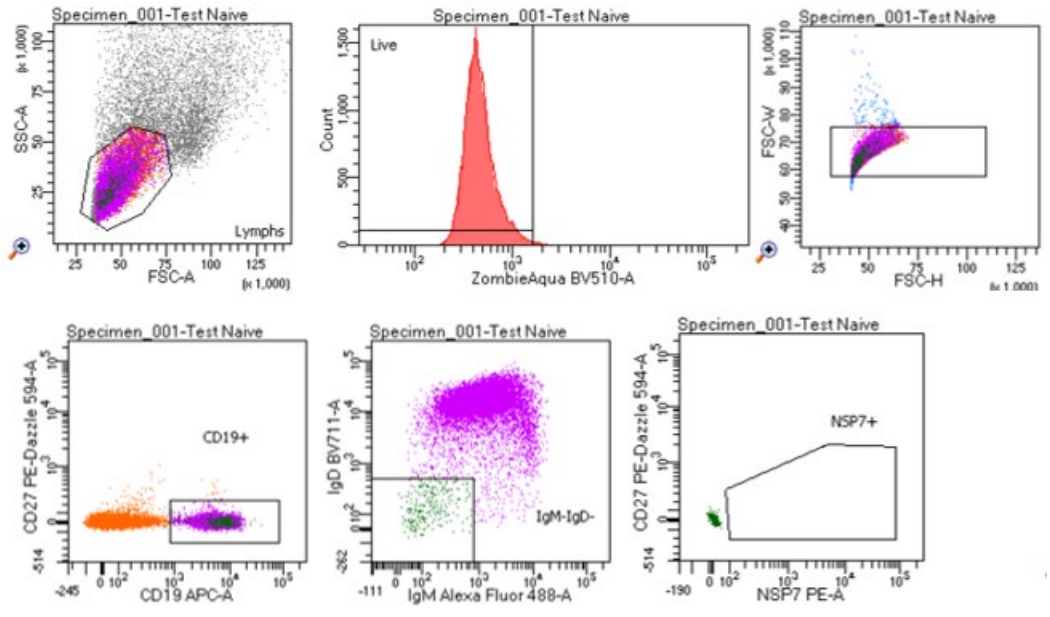

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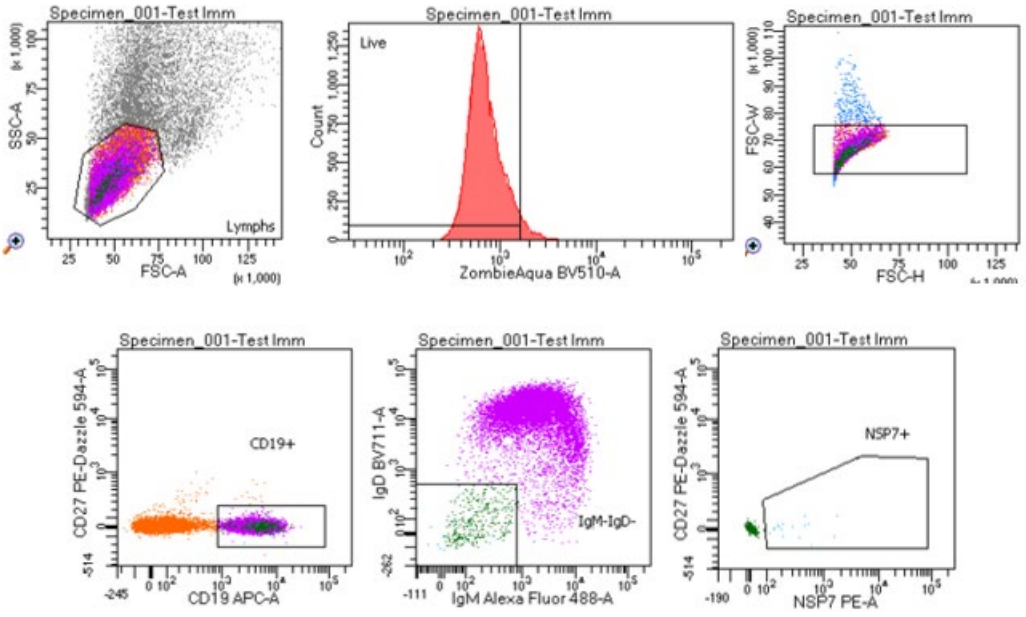


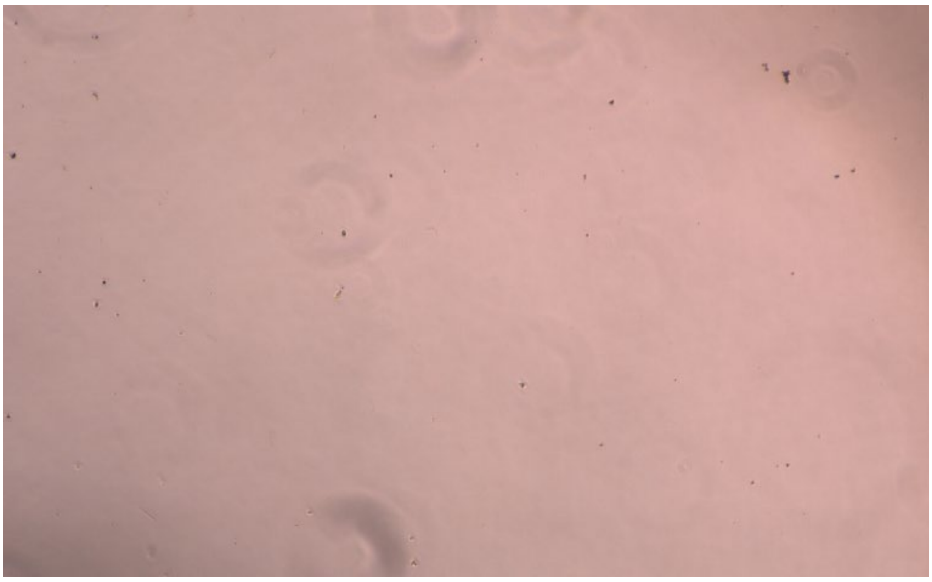
Figure 3.3. Antigen specific memory B cell sorting.

(A) gating strategy for selecting NSP7 specific IgG expressing memory B cells. (B) Naïve cell population stained with all the markers showing lymphocytes (lymphs) gated population, live cells followed by single cell population gating. Below CD19+ population, followed by gating of

IgM, IgD DN cells. lastly gating for NSP7 stained cells. (C) Test immunized population stained with all the markers in the same order as that of naïve sample.

Cell sorting by FACS indicated that although, there was no significant difference in the CD19+ or DN population between naïve and immunized samples, cells from naïve mouse did not show any staining for NSP7-546, whereas in test sample, 1-2% of DN population were positive for NSP7-AF546 staining, confirming that the antigen staining was selective, and no non-specific binding of NSP7-AF546 with memory B cells of naïve mice took place. For side-by-side comparison of the effect of antigen specific enrichment of memory B cells, two sets of cell population were sorted out from the immunized test sample. One that was total memory B cell population-CD19+ and DN, the other, which was NSP7 specific memory B cells- CD19+, DN as well as NSP7+. These sorted B cells were transiently cultured for 13 days with CD40L expressing recombinant 3T3-msCD40L fibroblast cells and 50ng/ml of recombinant mouse IL-2 (Sigma, cat#I0523-20UG) and IL-21 (Biolegend, cat#574504) in 96 well flat bottom plate. Each well was seeded with 25 NSP7+ B cells. For the culture of total memory B cells, 100 cells were added to each 96 well in order to increase the chance of each well obtaining a NSP7 specific cell. Few wells were seeded with B cells along with just IL-2 and IL-21 and no 3T3-msCD40L feeder cells (Figure3.4 A) to determine if the cytokines alone were enough or not to grow B cells in the absence of the feeder cells. At the end of 13 day, it was observed that B cells only grew and proliferated when both CD40L as well as cytokines were present (Figure 3.4 B), just the presence of cytokines alone was not sufficient.

A



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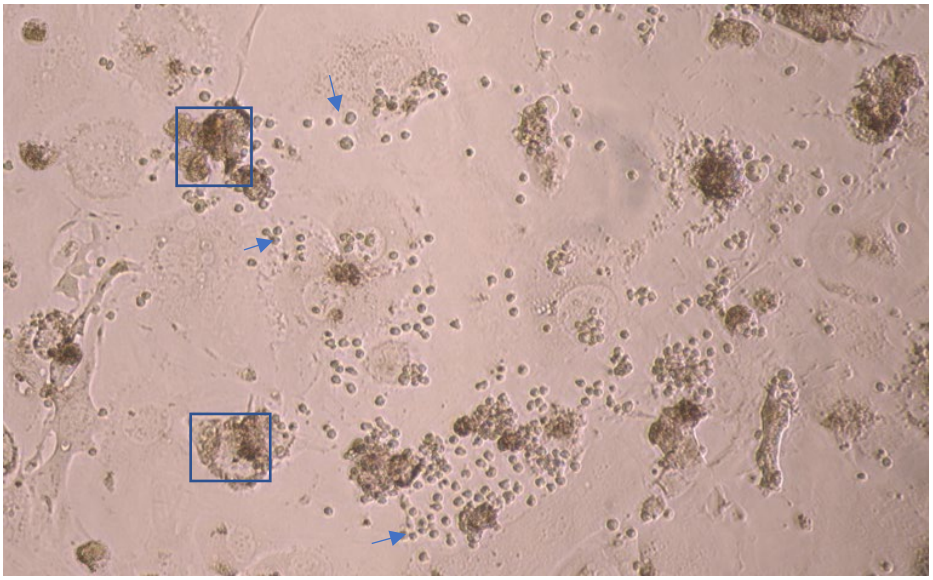


Figure 3.4. B cell co-culture.

(A) B cell cultured in medium with cytokine and no CD40L expressing cells. (B) B cells co-cultured with CD40L expressing cells and cytokines. Small dots indicated by the arrow are the expanding B cells, and apoptotic cells represented by the square box are the feeder cell. Images were taken at 10X magnification on day 13 after seeding the cells.

Supernatant from the cell cultures were taken to detect the presence of antibodies against NSP 7 by ELISA. It was observed that out of 30 different wells that were seeded with NSP7 specific memory B cells, except for one well, all of them were expressing high level of anti-NSP7 antibodies represented by most of the absorbance values above 4 OD as obtained in ELISA (figure 3.5). Compared to which, total memory B cell culture had only 6 wells positive for anti-NSP7 from a total of 180 wells. Indicating, that just by enriching the B cell population for antigen, the frequency of having positive B cell clones secreting antigen specific antibody was increased from 3.3% in total memory B cell population to 96.6% in NSP7 specific population.

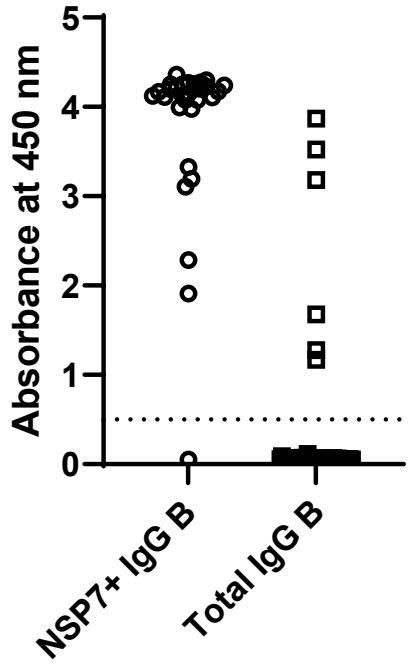


Figure 3.5. Frequency of B cell clones expressing anti-NSP7 antibodies.

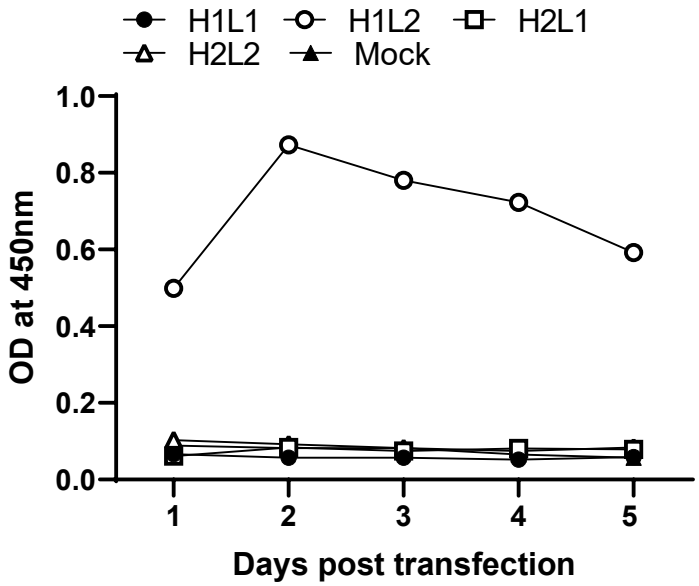
Dotted line at 0.5 OD indicates the cutoff of absorbance value for a sample to be positive.

One of the positive wells from NSP7 specific sorted cell culture was selected to isolate the antibody genes. Cells were lysed and total RNA was extracted from the sample. RNA was used to obtain cDNA for the variable region of both the heavy and light chain IgG gene. The cDNA was further PCR amplified and cloned into Topo pJet1.2 blunt end vector and six different colonies each for heavy and light chain were send for sequencing. After confirming the sequence and performing multiple sequence alignment by an online tool MUSCLE, it was concluded that the variable regions of heavy and light chains were from two different antibodies. The sequences were BLAST searched to annotate that the genes belonged to mouse IgG, following which variable regions genes were further cloned into two different pCI vectors containing constant region for heavy chain and light chain respectively. A total of four different full-length gene pCI constructs named as H1-heavy from clone 1, L1-light from clone 1, H2-heavy from clone 2 and L2-light from clone 2 were obtained. These vectors with full length heavy and light chain IgG sequences were transfected into HEK-293 T cells for expressing the whole antibody. To maintain the functionality of the antibody proper pairing of heavy chain with its cognate light chain is important, therefore four different combinations of heavy and light chains were tried as shown in Figure 3.6 A.

Supernatant from the transfected cells were collected from day 1 until day 5. On performing ELISA against NSP7 with the cell culture supernatants, it was observed that out of all the combinations, H1L2 was the only one expressing a functional antibody that detected and bound to NSP7 protein (figure 3.6). The level of antibody expression peaked on day 2 and 3 and decreased by day 5. Compared to which H1L1, H2L1, H2L2 or mock transfected cells had no signal at all, implying that overall, the procedure worked in mice. Functional antibodies specific to an antigen were expressed that detected the NSP7 protein.

combinations	Days post transfection (OD at 450nm)				
	1	2	3	4	5
H1L1	0.066	0.057	0.057	0.052	0.059
H1L2	0.499	0.873	0.780	0.723	0.592
H2L1	0.061	0.084	0.075	0.081	0.079
H2L2	0.103	0.092	0.082	0.075	0.084
Mock	0.089	0.082	0.082	0.065	0.057

A



B

Figure 3.6 Expression of functional antibody.

(A) table indicating OD values of different combinations of antibody gene transfected cell supernatant and mock supernatant from ELISA (B) graphic indicating the dynamics of antibody expression at different time point from Transfected or mock cells.

DISCUSSION

Monoclonal antibody generation by the process of antigen specific B cell co-culture was successfully optimized in the mice. The labeling of antigen showed specific activity and no naïve cells were stained for NSP7, which when used to enrich memory B cell population binding to NSP7 protein, enhanced the efficiency of anti-NSP7 expressing B cells in the culture. This shows that the simple method of incubating the protein with a commercially available fluorophore conjugation kit like that of AF-546 used in this study, is sufficient to label the protein. Even without the need of other complex methods like labelling using a biotin-streptavidin tetramer molecule [132], recombinant virus particles expressing fluorophore genes [133], or expression of recombinant protein together with the fluorophore [134] that sometimes can result in the change of antigen conformation and becoming unable to bind to the antibody or B cell receptor that otherwise would have detected. By using the feeder cells expressing CD40L and the costimulatory cytokines, the requirement of species-specific myeloma cell line was eliminated. The ligand and cytokines also enabled single B cells to proliferate and undergo clonal expansion [127] which increased the genomic content for the same monoclonal antibody. The increased amount of template obtained facilitated better amplification of the antibody genes. In future, same methodology can be used to try to make stable porcine monoclonal antibodies.

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