Development of efficacious replicon particle vaccines for swine influenza virus

by

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Swine influenza virus (SIV) continues to be endemic in the United States swine population. The clinical signs most commonly exhibited during SIV infections include acute respiratory disease with coughing, dyspnea, fever, anorexia and weight loss, nasal and ocular discharge, and lethargy.[2] Morbidity in infected herds is high (near 100%) while mortality is low (usually less than 1%) in the absence of concurrent infections and recovery usually begins 5-7 days after onset.[2] Despite low mortality, SIV can cause significant production losses as infection has been estimated to add two weeks to the time it takes a pig to reach market weight.[5] In addition to the economic and production losses due to influenza, the recent human influenza outbreak in 2009 has renewed concerns of the zoonotic potential of influenza viruses.

Influenza A viruses belong to the family *Orthomyxoviridae* and contain negative sense, single-stranded RNA genomes. The genome contains eight gene segments that encode for ten or eleven known proteins.[1] Currently, 16 different HA and 9 different NA subtypes have been identified in many different animal species.[3] Due to numerous subtypes and the segmented viral RNA genome, there is a large diversity among current influenza strains. This diversity occurs by two different methods, antigenic shift and drift. Antigenic shift, or genetic reassortment, occurs when two or more different influenza viruses co-infect the same cell and exchange gene segments during viral replication. Antigenic drift refers to the accumulated mutations that occur in the RNA viral genome and are most often identified in the HA and NA genes. Both antigenic shift and drift can result in decreased efficacy of current influenza vaccines. For this reason,

human influenza vaccines must be updated yearly to include temporally relevant influenza strains that offer the best chance of protection against seasonal influenza. Swine influenza vaccines must also be updated periodically to remain efficacious against the evolving diversity of currently circulating SIV strains in the United States swine population.

The first swine influenza virus was isolated in 1930[7] and is referred to as classical H1N1. This lineage of SIV was the predominant circulating virus in the US swine population until the 1997-98 influenza season when novel double and triple SIV reassortant viruses appeared.[5,11,12] These reassortant strains were of the H3N2 subtype, but only the triple reassortant H3N2 containing genes from human, avian, and swine influenza viruses spread efficiently in the swine population.[9] The triple reassortant H3N2 continued to undergo reassortment at a rapid pace, resulting in the appearance of several novel subtypes, namely reassortant H1N1 and H1N2 subtypes.[9,10] These viruses have undergone further reassortment with human influenza viruses, resulting in human-like H1N2 and H1N1 viruses in pigs.[8] Most notably, the pandemic H1N1 virus, which contains genes of swine origin, appeared in humans in spring 2009 and quickly spread worldwide.[4] The emergence of the pandemic H1N1 (pH1N1) influenza virus and the continuing shift and drift observed among SIV isolates demonstrates that the virus is constantly changing within the swine population, and that interspecies transmission can have devastating and costly consequences.

It has been suggested that the pH1N1 outbreak should prompt proactive research for the development of more advanced influenza vaccines rather than the reactive traditional preparation of human influenza vaccines.[6] Rappuoli et al. identified four

components required to develop a proactive influenza vaccine research program: adjuvants that increase cross-protection, conserved antigens, different vaccine platforms, and alternative vaccine delivery approaches.[12] Two of these components, the alphavirus replicon vaccine platform and conserved influenza antigens, are evaluated in this dissertation. In addition, these swine influenza vaccines were evaluated for efficacy and safety in swine.

The first paper presented in this dissertation describes the rapid development and efficacy of a recombinant HA subunit swine vaccine for protection against the 2009 pH1N1 influenza virus. This paper illustrates the speed with which a vaccine can be produced for an emerging disease using the alphavirus replicon technology.

The second paper examines the ability of an HA RP vaccine to be shed and spread among comingled cohorts, and whether the RP is capable of reverting to virulence in both host and non-host animals species. This paper also presents results obtained from immunogenicity and efficacy studies that were performed evaluating the same vaccine.

The third paper evaluates different RP vaccines against the pH1N1 influenza virus. First, an HA vaccine is evaluated against homologous pH1N1 challenge. Second, an NP vaccine is evaluated against heterologous challenge in an attempt to identify a broadly-protective vaccine that is capable of protecting against multiple SIV subtypes.

Dissertation Organization

This dissertation is organized in a journal paper or manuscript format. Chapter 1 includes a brief introduction to the influenza virus and briefly describes the journal papers and format of this dissertation. Chapter 2 provides a detailed literature review of

the alphavirus replicon vaccine technology utilized in the three journal papers. Chapters 3, 4, and 5 are manuscripts describing the research and results obtained by the primary research author, Ryan Vander Veen, along with the listed co-authors. Chapter 6 summarizes the general conclusions of the research followed by a brief acknowledgements section.

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CHAPTER 2: ALPHAVIRUS REPLICON VACCINES

A manuscript submitted to *Animal Health Research Reviews*

Ryan L. Vander Veen, D.L. Hank Harris, Kurt I. Kamrud

Abstract

The alphavirus replicon technology has been utilized for many years to develop vaccines for both veterinary and human applications. Many developments have been made to the replicon platform recently resulting in improved safety and efficacy of replicon particle vaccines. This review provides a broad overview of the replicon technology and safety features of the system and reviews the current literature of replicon particle and replicon-based vaccines.

Introduction

Traditionally, veterinary vaccines have consisted of either modified live or inactivated preparations. Modified live vaccines (MLV) have the possibility of reverting to virulence with subsequent spread among surrounding animals.[9,45] Inactivated vaccines often generate insufficient cell-mediated immunity required for protection so must be combined with adjuvants that are able to induce the required immune response.[46,47] Thus, there has been a focus on "second-generation" vaccines, some of which have already been licensed for commercial veterinary use.[46]

Alphavirus replicon-based vaccines represent a viable option for next-generation vaccine development. To date, alphavirus replicon-based vaccines have not been approved by any government regulatory agency for use in animals or humans. Alphavirus replicon particles (RP) are single-cycle, propagation-defective particles that

are not able to spread beyond the initial infected cells. Replicon particles are produced by removing the alphavirus structural genes from the replicon RNA vector and replacing with a heterologous gene(s) of interest. The helper genes can be provided *in trans* along with replicon RNA and co-transfected into permissible cells, resulting in the packaging of the replicon RNA. Replicon particle vaccines have been evaluated in many different species of animals as well as humans with a proven record of safety and efficacy. These vaccines are capable of inducing robust and balanced immune responses and offer many other advantages that ideal vaccines possess.

Alphavirus biology

The *Alphavirus* genus belongs to the *Togaviridae* family and contains 28 virus species.[21] Alphaviruses are positive-sense, single-stranded RNA viruses with a genome of approximately 11.5kb in length. The positive-sense genome contains two open reading frames (ORFs) and encodes four nonstructural proteins and five structural proteins.[64] The 5' ORF encodes four nonstructural proteins (nsp1-4) and the 3' ORF encodes the virus structural proteins (capsid and glycoproteins (E3, E2, 6K and E1)).[57,64] The nonstructural proteins are translated from the positive-sense genomic RNA and function to transcribe full-length negative-sense RNA (Figure 1). Translation of the nsp1-3 polyprotein is terminated by an opal stop codon located between nsp3 and nsp4; the polyprotein nsp1-4 is produced when translational readthrough occurs at the nsp3-4 junction.[35] The negative-sense RNA is a template for both additional genomic RNA as well as 26S subgenomic mRNA. The 26S promoter is located between the two ORFs on the negative-sense RNA and is recognized by the nonstructural proteins for transcription

of a subgenomic mRNA, from which the structural proteins are translated. This 26S mRNA is produced in 10-fold molar excess when compared to genomic RNA.[64] The structural proteins are translated from the subgenomic 26S mRNA as a polyprotein that is subsequently co-translationally and post-translationally cleaved to release the capsid protein and the two mature envelope glycoproteins (E1 and E2).[29] Foreign genes of interest can be inserted in the place of alphavirus structural genes in cDNA clones generating a self-replicating RNA (replicon) capable of expressing the foreign gene when introduced into cells. The self-amplifying replicon RNA directs the translation of large amounts of heterologous protein in transfected cells, reaching levels as high as 15-20% of total cell protein.[56] The replicon RNA can be packaged into RP by supplying the structural genes *in trans* in the form of capsid and glycoprotein helper RNAs (Figure 2).[33,56] When the helper and replicon RNAs are co-transfected into permissible cells, the replicon RNA is efficiently packaged into single-cycle, propagation-defective RP that are morphologically indistinguishable from native alphaviruses (authors' unpublished observation). Importantly, only the replicon RNA is packaged into RP, as the helper RNAs lack the packaging sequence required for encapsidation. Therefore, the resulting RP are propagation-defective and are incapable of producing progeny particles or virus.

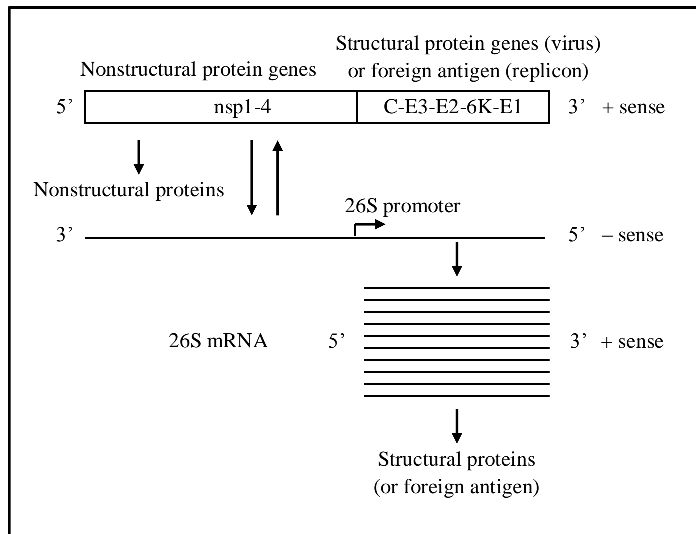

Figure 1. Alphavirus genome organization and replication strategy.

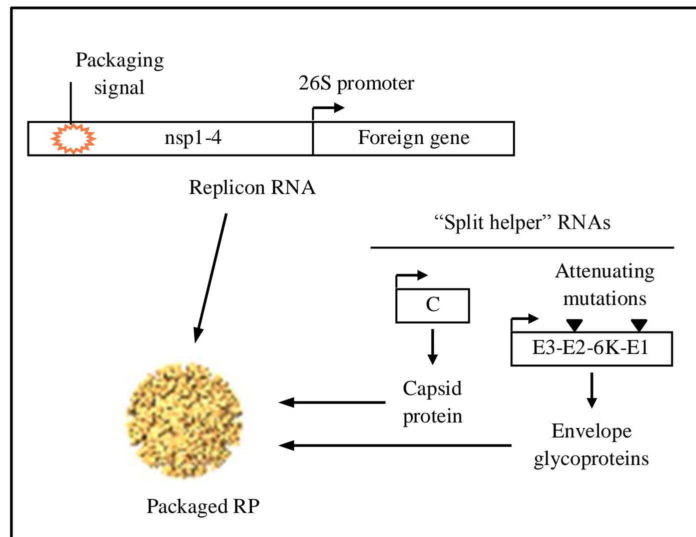

Figure 2. Alphavirus replicon particle vaccine and packaging system.

There are several features of alphavirus RP that make them attractive for vaccine development: 1) a proven record of safety; 2) high expression levels of heterologous genes; 3) dendritic cell tropism; 4) protective and balanced immune responses; 5) multivalent vaccine construction; 6) resistance to anti-vector immunity; 7) commercial vaccine production; and 8) differentiation of infected and vaccinated animals (DIVA).

Safety

Replicon vectors have been developed from several different alphaviruses, including Venezuelan equine encephalitis virus (VEEV), Sindbis (SIN), and Semliki Forest virus (SFV).[10,41,56] Replicon vectors lack the alphavirus structural protein genes but retain the nonstructural genes and the 26S subgenomic promoter. Early production of RP were hampered by recombination events that resulted in the generation of replication-competent virus (RCV)[10,57,70]; first generation helper RNAs encoded the capsid and glycoprotein genes on the same RNA molecule, and thus only required one recombination event to create RCV.[5,20,56] The probability of this event occurring was greatly reduced by separating the helper RNAs onto two separate RNAs ("split helper" system). Pushko et al. were able to demonstrate increased safety of the system by decreasing recombination events leading to the production of infectious virus when utilizing the split helper system.[56] When both helper genes (capsid and glycoprotein) were present on a single RNA and co-electroporated into cells with replicon RNA expressing a heterologous gene, infectious virus was typically recovered, even to levels as high as $2x10^5$ PFU/ml.[56] However, when the helper RNAs were split onto two separate RNA molecules, there was no recovery of infectious virus by either plaque assay, blind passaging, or intracerebral inoculation of mice.[57] Similar split helper systems have also been developed for SIN and SFV helper RNAs resulting in no recovery of infectious virus.[18,63]

The split helper system greatly reduces the occurrence of RCV, as separation of the helper RNAs requires two independent recombination events to occur for generation of RCV.[56,62] Initially, helper RNAs were designed to contain a 26S promoter

downstream of the 5' untranslated region because of the significant impact the promoter has on the production of high levels of subgenomic transcripts. However, a recent study has demonstrated that the 26S promoter is not required for functional helper RNAs.[30] Removal of the 26S promoter results in helper RNAs that are not independent transcriptional units, and further reduces the probability of functional recombinations between the replicon and helper RNAs. In the same study, a stop codon was introduced at the 3' end of the capsid gene in place of the chymotrypsin-like cleavage site.[30] This mutation negates the cleavage activity of the capsid protein, adding another safeguard against functional recombination. Thus, helper RNAs lacking 26S promoters that have been manipulated to include a capsid stop codon have a reduced probability of functional recombination than the standard split helper RNA system.[30] The introduction of the split helper system and subsequent modifications were significant advances in replicon technology that have facilitated RP vaccine evaluation *in vivo* without risk of reversion to virulence.

Human preclinical evaluation of cytomegalovirus (CMV) and influenza RP vaccines has been completed with no adverse effects reported in toxicology studies. An HA/NA influenza RP vaccine was administered to white rabbits four times with no toxic side effects and no relevant clinical parameter differences observed between RP and placebo vaccinated animals.[24] Similar results were observed following evaluation of a candidate CMV RP vaccine in rabbits.[59] This CMV vaccine has recently been evaluated in a Phase I human study where the vaccine was well tolerated with only mild to moderate local reactogenicity and minimal systemic reactogenicity even after three doses.[7]

Other studies have used the mouse model of intracranial injection to demonstrate the safety of RP. Intracranial (IC) injection of VEEV RP resulted in only transient pathology (1-2 days) and weight loss (1 day) with a rapid return to pre-injection status.[37] The neurovirulence of both SIN and SFV RP expressing LacZ have also been evaluated following IC inoculation. Beta-galactosidase activity was detectable in brains for 14-28 days, but no morbidity, neuropathology, or loss of motor skills was observed in either study, indicating a lack of reversion to the parental virus strains.[2,43] A biodistribution study done with the same SFV RP system demonstrated that following IC injection there was no evidence of RP RNA co-localization to either the liver or the brain after 15 days, and no abnormalities were observed during histopathological examination.[50] These results correlate with safety studies we have completed in pigs and mice following injection with a RP vaccine expressing an H3 influenza gene. The results demonstrate a lack of shed and spread of RP RNA and a lack of reversion to virulence following vaccination (manuscript submitted).

The current molecular features of the RP system result in safe vaccines. However, if in such a rare circumstance the perfect pair of recombination events did occur in the exact order and positions needed,[30] the resulting RCV would theoretically be no more virulent than the parent strain being used as the vaccine vector. The attenuated VEEV strain TC-83 (commonly used in replicon vector development and production) has been used extensively to vaccinate military personnel and lab workers against VEEV infection.[1,11] A retrospective study of hundreds of humans who received the vaccine from 1976 to 1990 indicated that TC-83 vaccine caused some transient reactions, but no serious sequelae were reported.[53] This strongly suggests that if multiple improbable

functional recombination events were to occur during RP production, the resulting recombinant virus would theoretically be no more virulent than the infectious parent strain and thus would not have serious public health consequences.

High Expression Levels of Heterologous Genes

Pushko et al. demonstrated that transfection of several cell lines with RP expressing the Lassa virus N protein resulted in expression levels of nearly 20% of total cell protein.[56] Kamrud et al. engineered the VEEV replicon to allow further optimization of protein yield and replicon packaging efficiency.[32] Internal ribosome entry site (IRES) elements were inserted into the replicon vector downstream of the 26S promoter to allow for cap-independent translation of heterologous genes. In addition, random nucleotide sequences of varying lengths were inserted between the 26S promoter and the IRES element. When compared to null replicons (no IRES) the spacer-IRES replicons expressed protein in some instances at >50 fold increases.[32] Thus, by varying the length of the spacer sequences used in conjunction with IRES elements, replicons expressing the highest levels of heterologous genes or resulting in the highest RP yield can be readily identified for vaccine evaluation.

Dendritic Cell Tropism

A robust immune response is dependent on accurate and rapid presentation of the antigen to immune effector cells. Dendritic cells (DC) are considered professional antigen presenting cells so vaccines that target these cells should induce robust and balanced immune responses. Inoculation of mice with VEEV RP revealed Langerhans cells (DC located in the skin) as the initial cell set to be infected.[44] In humans, VEEV RP

have a selective human myeloid DC tropism and these DC retain the capacity to acquire the mature phenotype upon migration to the local draining lymph node.[52] SFV has also been shown to infect Langerhans cells and subsequently migrate to the local lymph node.[28] A single amino acid substitution in the E2 glycoprotein of the SIN replicon vector significantly increased the affinity of the particle for human DC, resulting in an increase of major histocompatibility complex (MHC) class II molecules, CD86, and IFN-γ secreting cells.[19] This natural or enhanced DC tropism of alphavirus RP results in a balanced and protective immune response following RP vaccination.

Protective Immunity

The alphavirus replicon system has been used to develop efficacious RP vaccines for both human and veterinary applications. Influenza RP vaccines have been evaluated in chickens, pigs, and humans. Complete protection against lethal H5N1 avian influenza challenge was demonstrated in 2 week old chickens that received a single dose of RP vaccine expressing the homologous HA gene.[61] Recent reports also demonstrate that protective HI responses are elicited in young pigs following HA RP vaccination.[8,17] Following homologous challenge, there was a significant decrease in nasal shedding, viral load, rectal temperatures, and pulmonary pathology in HA RP vaccinated animals compared to placebo controls.[8] The alphavirus replicon system was also used to rapidly produce a recombinant HA protein vaccine in response to the pandemic H1N1 influenza outbreak in 2009 with similar protection observed following homologous challenge.[69] Other influenza gene candidates have also been evaluated in the RP system including neuraminidase (NA) and nucleoprotein (NP) with varying results.[8,24,66] Preclinical

evaluation of HA and NA RP vaccines have been completed for human trials with no adverse effects observed following toxicology and safety testing, and robust humoral and cellular responses were elicited in mice, rabbits, and rhesus macaques.[24] In addition to evaluation as influenza vaccine candidates, RP not expressing any heterologous genes (null RP) are able to act as adjuvants and enhance the immunogenicity and efficacy of a trivalent inactivated influenza vaccine in rhesus macaques.[12]

In addition to influenza, replicon particle vaccines have also been evaluated against several other animal diseases of veterinary importance. Balasuriya *et al.* used VEEV RP expressing the G_L and M proteins of equine arteritis virus (EAV) and demonstrated that these two major envelope proteins are necessary as a heterodimer for the induction of EAV neutralizing antibodies in mice.[4] Further, only horses vaccinated with the G_L/M RP vaccine were protected against virulent challenge while horses receiving RP expressing only the G_L or M monomers were not protected from EAV.[3] Similar research has been completed evaluating a related *Arterivirus*, porcine reproductive and respiratory syndrome virus (PRRSV), where the GP5/M heterodimer has also been shown to be important for neutralizing antibody induction.[25-27] Replicon particle vaccines expressing the PRRSV GP5/M heterodimer have been evaluated in swine with a reduction in viremia observed post-challenge.[48,49] A recent study evaluated RP vaccines that encoded either the glycoproteins of Hendra or Nipah viruses,[15] diseases of both veterinary and public health importance. These vaccines were able to induce cross-reactive neutralizing antibodies to both viruses, suggesting that a single vaccine against both viruses may be possible.[15]

In addition to alphavirus-based RP vaccines, alphavirus vectors can be designed to launch a self-replicating replicon RNA from a DNA plasmid *in vivo*. Replicon DNA vaccines have been developed for SIN, SFV, and VEEV.[6,16,36,42] The first step of expression involves RNA polymerase II-initiated transcription of replicon RNA from plasmid DNA in the transfected cells. Currently, the CMV immediate-early promoter is most frequently used.[42] The second step of expression occurs when the replicon RNA enters the cytoplasm and the heterologous gene of interest is amplified from the native alphavirus 26S subgenomic RNA promoter. Previous SFV- and SIN-based replicon DNA vaccines have been shown to be immunogenic in small animal models.[6,22] A VEEV replicon-based DNA plasmid consistently expressed 3- to 15-fold more protein *in vitro* and increased humoral responses by several orders of magnitude over a conventional DNA vaccine.[42] As the transcribed replicon RNA is self-amplifying, increases in cellular and humoral responses were also observed when 100-fold lower doses of VEEV DNA was used compared to conventional DNA. Additionally, when the VEEV DNA vaccine was used as a prime and VEEV RP expressing the same heterologous gene was given as the boost dose, both humoral and cellular immunity were increased significantly compared to VEEV DNA alone.[42]

Similar replicon-based DNA vaccines have recently been evaluated in mice and pigs. A SFV replicon-based DNA vaccine has been produced expressing the E2 glycoprotein of classical swine fever virus (CSFV). Pigs vaccinated with this DNA vaccine elicited low levels of neutralizing antibodies one week post-booster vaccination but exhibited decreased clinical symptoms and reduced viremia following homologous challenge when compared to control pigs.[40] When evaluated in a mouse model, this

vaccine elicited a specific lymphoproliferative response and an increase in IFN-γ and IL-4 CSFV-specific secretion.[73] When the CSFV DNA vaccine was used in a prime/boost regimen with a recombinant adenovirus expressing the homologous E2 glycoprotein a significant increase in pre-challenge neutralizing antibody titers was observed with subsequent protection against heterologous CSFV challenge.[72] A SFV replicon-based DNA vaccine expressing the 1BCD gene of swine vesicular disease virus has also been shown to elicit neutralizing antibodies and lymphocyte proliferation in both guinea pigs and swine.[65] A SIN virus replicon-based DNA vaccine encoding the rabies virus glycoprotein G induced higher levels of humoral and cell-mediated immunity in mice than the conventional rabies DNA vaccine and comparable to the inactivated commercial rabies vaccine.[60] All of the replicon DNA and inactivated virus vaccinated mice were completely protected against lethal challenge while some mice receiving the conventional DNA vaccine did not survive.[60] All of these results indicate that the alphavirus replicon technology is flexible and that replicon RNA can be delivered to the host by several different methods. Also, replicon technology can be used in conjunction with other recombinant systems to produce more efficacious vaccine regimens.

Multivalent Vaccines

One of the advantages of the alphavirus replicon system is that the vector can be genetically modified to express several different genes either from the same or different pathogens. This can be accomplished via several methods, but the most common method is insertion of additional 26S promoter sites downstream of the non-structural genes. However, not all proteins are expressed at equimolar levels in this design and can depend

on the number of genes or gene position in the replicon, so each replicon must be empirically evaluated to determine optimum protein expression.[58] Mice that received RP vaccines expressing different combinations of pp65, IE1, and gB proteins under control of three different 26S promoters developed high titers of neutralizing antibody and antigen-specific T-cell responses against CMV.[58] Balasuriya et al. produced an RP vaccine co-expressing the G_L/M heterodimer that was required for protection against EAV.[3] Replicon particle vaccines expressing genes from two different pathogens are also able to induce protection against subsequent challenge. An RP vaccine co-expressing the glycoprotein genes of both Ebola and Lassa viruses protected guinea pigs from challenge with both viruses.[55] The results obtained were the same as those achieved with RP vaccines expressing only one of the viral glycoproteins, indicating that protective immune responses can be induced against multiple and individual vaccine antigens at similar levels. In other studies the authors have simply mixed different monovalent RP vaccines together prior to injection rather than producing the vaccine using a bivalent approach with additional 26S promoters.[23,38] Similar to the previous results, a specific immune response was elicited in mice to each individual RP antigen including Marburg virus, anthrax, and botulinum neurtotoxin, and protection was demonstrated following challenge with *Bacillus anthracis* and botulinum neurotoxin A and C.[38] The level of protection against *B. anthracis* induced by this multivalent vaccine formulation was similar to the protection demonstrated by vaccination with a monovalent anthrax RP vaccine.[39] Hooper et al combined individual RP expressing four different smallpox virus antigens and demonstrated protection from lethal monkeypox virus challenge in cynomolgus macaques.[23] Taken together, all of these results indicate that

multivalent RP vaccines, regardless of the method of production, are able to induce balanced antigen-specific immune responses and can protect against multi-agent challenge. These results may be important for decreasing the number of injections required for protection against several diseases or serotypes of a single disease and also for decreasing the cost of RP vaccine production.

Resistance to Antivector Immunity

As the alphavirus structural genes are not packaged and thus not expressed following vaccination, antivector immune responses are minimal. This lack of antivector immunity allows for multiple vaccinations of the same individual with either the same RP vaccine or different RP vaccines against multiple pathogens. Pushko et al. first demonstrated that RP could be used for sequential immunization by vaccinating mice with two doses of RP expressing the N protein of Lassa virus and then sequentially vaccinating the same mice with two doses of HA RP.[56] These mice developed positive serum antibody responses against both antigens. Additionally, vaccinated mice were protected against influenza challenge, indicating that the Lassa N immune response did not interfere with subsequent influenza vaccination and the influenza-specific immune response. These same results have been achieved following vaccination with RP expressing the HA protein from two different subtypes of influenza (authors' unpublished results). Ferrets were first immunized with an H3 RP vaccine and subsequently protected against homologous H3N2 influenza challenge. These protected ferrets (pre-immunized) were then vaccinated with H1 RP along with a group of naïve (non-H3 RP vaccinated) animals. Both the pre-immunized and naïve ferrets that received the H1 RP vaccine

developed equivalent H1N1 hemagglutination inhibition (HI) serum antibody titers. These studies confirm that RP vaccines can be used sequentially without an inhibitory effect on vaccine efficacy. Other recombinant vaccines are often hindered by antivector immunity and it has been suggested that recombinant adenovirus vaccination regimens should include two heterologous vectors to avoid antivector immunity.[67]

Commercial Vaccine Production

Many diseases that have not yet had efficacious vaccines developed against them are in the NIH Risk Group 3 (ie. highly pathogenic avian influenza H5N1 and human immunodeficiency virus (HIV) types 1 and 2) or on the APHIS/CDC Select Agent List (ie. Botulinum neurotoxins, Ebola and Marburg viruses, *B. anthracis*, Hendra and Nipah viruses, foot-and-mouth disease virus (FMDV), and CSFV). Thus, all of these pathogens require either BSL3 or BSL4 production facilities for traditional vaccine production. This requirement has almost certainly been an impediment in the development of some of these vaccines. In addition, the alphavirus VEEV is also listed as a select agent, except for the TC-83 attenuated vaccine strain. Thus, the TC-83 infectious clone can be utilized in the replicon system with the protective genes of the aforementioned select agents at low biocontainment levels for research and production. Since replicon-based vaccine production does not require growth of the pathogenic organism, select agent replicon-based vaccine development and production can occur in low biocontainment production facilities with no special biosecurity required. The capability to produce select agent vaccines in such production facilities is a huge advantage of the replicon system and has aided the development of these crucial vaccines for both humans and animals. The

attenuating determinants of TC-83 virus attenuation have been well defined.[34,35] Because of the attenuated safety profile of TC-83 it has been recently developed as a replicon vector.[8,17,23,31] A recent study compared a TC-83-based RP vaccine against a different RP vaccine (V3014-based, vaccine produced in BSL3 facilities) in pigs. Both RP vaccines expressing H3N2 HA were able to elicit the same antibody response in pigs demonstrating that there is no difference in immunogenicity between RP vaccines produced using these two VEEV replicons.[17]

Another advantage of RP vaccines is that many different cell lines are permissive to alphavirus infection allowing a variety of different cells to be evaluated for maximum yields. Vero and baby hamster kidney cells are most commonly used for RP production, but chinese hamster ovary (CHO), primary chicken (CEF) and duck (DEF) embryo fibroblasts, 293, and 293T cell lines have also been utilized for RP production (authors' unpublished results).[56] This long list of cells is in contrast to pathogenic virus growth which can usually only occur optimally in one cell line. As in traditional vaccine production, these cells can be grown in large quantities using large-scale bioreactor microcarrier or suspension systems allowing efficient scale-up possibilities for RP production. There is also a report of stably transfected cell lines that have been developed to constitutively express the helper RNAs needed for RP production.[54] Similar to the split helper RNA system described above, only cell lines containing the structural protein genes on separate RNAs resulted in no recovery of RCV.[54]

DIVA Capability

One of the attributes of a good next-generation vaccine should be the capability to distinguish infected from vaccinated animals (DIVA). The DIVA concept relies on the principle that a vaccinated animal will have a different immune response than an animal that is infected with the wild-type pathogen and that this immune response is readily detectable by some immunoassay. These DIVA vaccines become increasingly important when considering diseases that are not currently present in disease-free status countries (ie. FMDV and CSFV) or for disease eradication and intense surveillance programs. In the case of FMDV, the current control policy has been primarily one of slaughtering the infected and contact animals.[68] This policy could have a huge detrimental impact on domestic livestock production and potential export ramifications. Following the 2001 FMDV outbreak in the UK there has been a growing demand for FMDV vaccination following an outbreak in order to reduce the large-scale slaughter of animals for control of the virus.[68] Current FMDV vaccine research is focused on development of DIVA vaccines expressing the capsid proteins, including FMDV replicon-based vaccine research (authors' unpublished results).[46,71]

Vaccines that have DIVA capabilities are also important for the control and eventual eradication of current endemic infectious diseases. Porcine reproductive and respiratory syndrome virus (PRRSV) is endemic to the United States swine population and continues to have a huge economic impact.[51] Current diagnostic assays for PRRSV target antibodies directed towards the nucleocapsid (N) protein. Antibodies to the N protein have been shown to be non-neutralizing,[14] and a protective vaccine need not include this protein to be efficacious. Thus, RP vaccines expressing any combination of

PRRSV structural glycoproteins would therefore be able to differentiate vaccinated from naturally infected animals. Similarly, a current ELISA for detecting influenza is based on NP antibody detection.[13] Replicon particle vaccines expressing only the HA protein would therefore not induce a detectable immune response when used in conjunction with this diagnostic assay. Therefore, alphavirus-based vaccines offer DIVA capabilities that can be important in different disease situations.

Conclusions

Research into the potential of alphavirus replicon-based vaccines has been ongoing for more than 20 years. Significant advancements have been made since these vectors were first used for the expression of heterologous genes. Improvements in both safety and the replicon vector design have significantly advanced the field of replicon-based vaccines. Both RP and replicon DNA vaccines have demonstrated robust and balanced immune responses with subsequent protection against a variety of diseases that have implications for both veterinary and human health. Thus, the alphavirus replicon technology offers great potential for the next generation of animal and human vaccines.

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CHAPTER 3. RAPID DEVELOPMENT OF AN EFFICACIOUS SWINE VACCINE FOR PANDEMIC H1N1

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Abstract

Recombinant hemagglutinin (HA) protein from a pandemic H1N1 influenza strain was produced using an alphavirus replicon expression system. The recombinant HA vaccine was produced more rapidly than traditional vaccines, and was evaluated as a swine vaccine candidate at different doses in a challenge model utilizing the homologous influenza A/California/04/2009 (H1N1) strain. Vaccinated animals showed significantly higher specific antibody response, reduced lung lesions and viral shedding, and higher average daily gain when compared to non-vaccinated control animals. These data demonstrate that the swine vaccine candidate was efficacious at all of the evaluated doses.

Introduction

The recent outbreak of pandemic H1N1 (pH1N1) in the global human population highlights the zoonotic potential of influenza viruses. The current pH1N1 virus has been shown to contain genes of swine origin.[4] Even before the current pandemic, numerous cases of zoonotic transmission of swine influenza viruses to humans have been

identified. A review of the literature in 2006 identified 37 civilian cases and 13 military cases of human influenza associated with swine influenza strains, spanning from 1958-2005.[12] Fourteen percent of these cases were documented as fatal. A more recent study reviewed reported cases of triple-reassortant swine influenza subtype H1 in humans from 2005-February 2009.[17] They found 11 sporadic cases, and all 11 patients recovered after showing clinical influenza symptoms. Nine of these 11 patients had known exposure to pigs, most of which were ill, either at agricultural fairs or at hog farms. These results mirror studies showing increased antibody titers to swine influenza viruses among hog farm workers and family members.[5,13] These studies provide evidence that human and swine interaction can result in the creation of pandemic influenza viruses, and thus the need for efficacious swine influenza vaccines. Pandemic H1N1 vaccination can reduce clinical disease in pigs, and may reduce transmission among the swine population and decrease the zoonotic potential.

Many vaccines have been evaluated using alphavirus replicon technology.[14] In this study, the alphavirus replicon is derived from the TC-83 strain of the alphavirus Venezuelan equine encephalitis virus (VEEV). In a previous study, a VEEV replicon vaccine expressing the HA gene from a human H5N1 isolate protected chickens from lethal challenge.[16] Recently, our group became the first to evaluate VEEV replicon particle vaccines in swine.[3] However, no studies have been published using replicon-expressed recombinant proteins as vaccine candidates for swine. The objective of this study was to evaluate replicon-expressed recombinant pH1N1 HA protein as a swine vaccine in a vaccination-challenge model.

Materials and Methods

Pandemic H1N1 HA replicon subunit vaccine production

The Influenza A/California/04/2009 hemagglutinin (HA) nucleotide sequence was retrieved from the Global Initiative on Sharing Avian Influenza Data (GISAID) database. The gene was synthesized by a commercial company (DNA2.0, Menlo Park, CA, USA) with unique AscI and PacI restriction sites engineered at the 5' and 3' ends, respectively. The HA gene was cloned into the AscI/PacI sites of the pVEK (TC-83) replicon vector[7] and an optimized construct was selected as previously described.[9] The HA gene was then sequenced to ensure the proper sequence was maintained throughout the cloning process. RNA transcripts were produced in vitro as previously described.[9] Replicon RNA was mixed with Vero cells in electroporation cuvettes and pulsed. Cells were incubated overnight and then lysed using RIPA buffer (Pierce, Rockford, IL, USA). Resulting lysate was tested for protein expression by Western blot and HA protein concentration was determined by a pH1N1 HA-specific ELISA. Lysate was diluted to the specified HA concentration and vaccine was adjuvanted with Emulsigen-D (MVP Technologies, Omaha, NE, USA).

Western blot analysis

Vero cell lysate containing recombinant HA protein was separated by running on a 12% SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA) and was then transferred to a PVDF membrane (Invitrogen, Carlsbad, CA). The ladder used was the SeeBlue Plus2 Pre-Stained Standard (Invitrogen, Carlsbad, CA, USA). After transfer, membrane was blocked with 5% non-fat dry milk at room temperature. Membrane was incubated with polyclonal anti-H1N1 antibody for two hours, washed three times, followed by

incubation with goat anti-swine IgG horseradish peroxidase conjugate (ImmunoJackson Research Laboratories, Inc, West Grove, PA, USA) for one hour, and washed three times. Detection was performed using TMB substrate (KPL, Gaithersburg, MD, USA).

Direct antigen capture ELISA

Unknown samples, negative controls, and purified pandemic H1 protein (Protein Sciences, Meriden, CT, USA) were directly captured to NUNC Maxisorp (Rochester, NY, USA) 96-well microplates by diluting with capture buffer (50 mM Carbonate/Bicarbonate, pH 9.6) and incubated overnight at 4°C (100 µl/well). The microplates were washed four times with wash buffer (20 mM Phosphate Buffered Saline, 0.05% Tween-20, pH 7.2). The plates were blocked with 1.25% non-fat dry milk in capture buffer for 1 hour at 37°C (200 µl/well). After four washes, polyclonal anti-H1N1antibody was added to wells (100 µl) and incubated for 1 hour at 37°C (diluted 1/500 in wash buffer containing 1.25% NFDM). Following four washes, goat ant-pig IgG-HRP labeled (Jackson ImmunoResearch, West Grove, PA, USA) was added to the wells (100 µl) and incubated for 1 hour at 37°C (diluted 1/2000 in was buffer containing 1.25% NFDM). Four final washes were performed prior to the addition of 100 µl of TMB substrate (KPL, Gaithersburg, MD, USA) and incubation at 37°C for 20 minutes. Absorbance values were measured at 620 nm and a standard curve was plotted with the purified pandemic H1 protein. Linear regression analysis of the standard curve was used to calculate the pandemic H1 concentrations in the unknowns.

Animal study

Pigs free of swine influenza virus (SIV) and porcine reproductive and respiratory syndrome virus (PRRSV) were obtained at three weeks of age. Pigs were randomized

and separated into 4 groups of 5 pigs each (Table 1). Prior to vaccination, serum was collected and tested by the homologous hemagglutination inhibition (HI) assay against the pH1N1 A/California/04/2009 influenza strain to confirm negative antibody status. Sera were collected throughout the study and tested by this same HI assay to monitor seroconversion post-vaccination. A prime/boost vaccination schedule was followed. The first dose of vaccine was given to pigs at approximately 4 weeks of age on day 0. On day 21 pigs received booster vaccination, with challenge on day 47 and necropsy on day 52. Pigs received either phosphate buffered saline (PBS) (Placebo, Group 1) or different concentrations of pandemic H1 HA recombinant protein (Groups 2-4, Table 1). Pigs were challenged intratracheally with virulent A/California/04/2009 influenza virus (CDC# 2009712047) at a dose of $2x10^5$ $TCID_{50}$. Nasal swabs were collected daily for live virus isolation beginning on day of challenge and continuing until study completion 5 days post-challenge. Pigs were weighed immediately before challenge and again at necropsy for determination of average daily gain (ADG). At necropsy, gross lung lesion consolidation was determined by a board-certified pathologist. Lung tissue was fixed in formalin for SIV immunohistochemistry (IHC) and histopathological analysis. Bronchoalveolar lavage fluid (BALF) was collected from lungs for live virus isolation. This animal study was approved by the Iowa State University Institutional Animal Care and Use Committee.

Hemagglutination inhibition (HI) assay

Antibodies to influenza virus were measured by HI assay run by the University of Minnesota Veterinary Diagnostic Laboratory following standard laboratory protocol. Briefly, sera were treated with receptor-destroying enzyme, heat inactivated, adsorbed

with 20% turkey erythrocytes, and centrifuged. Supernatants were then serially diluted in V-shaped well microtiter plates with an equal volume containing 4-8 agglutinating units of A/California/04/2009 virus and plates were incubated at room temperature before addition of 0.5% turkey erythrocytes. Titer was defined as the reciprocal of the maximal dilution at which hemagglutination was inhibited.

Gross lung lesion scoring, histopathology, and SIV immunohistochemistry

A single board-certified veterinary pathologist who was blinded to group treatments, performed gross lung scoring, histopathological analysis, and SIV Immunohistochemistry (IHC) analysis. At necropsy, each lung lobe affected by pneumonia was visually estimated, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume.[6] Tissue samples from the trachea and all lung lobes were collected and fixed in 10% formalin. Tissues were routinely processed and stained with hematoxylin and eosin. Lung samples were scored according to the method used by Vincent et al.[20] Swine influenza virus IHC was done according to the method described by Vincent et al.[21] All tissue preparation and staining was done by the Iowa State University Veterinary Diagnostic Laboratory.

Live virus isolation

Live virus titers were determined from nasal swabs and live virus isolation performed on BALF samples. Briefly, nasal swabs and BALF samples were thawed and centrifuged to remove cellular debris. The resulting supernatant was diluted 10-fold in 96 well plates in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 1% antibiotic-antimycotic (Gibco, Carlsbad, CA, USA) and 1% L-

glutamine (Mediatech, Manassas, VA, USA). After dilutions were made, 100µl was transferred from each well into respective wells of a 96 well plate containing a monolayer of swine testicle (ST) cells. Plates were incubated at 37°C until no further CPE was observed, typically 3-5 days. Wells displaying CPE were considered positive, and titers were calculated using the $TCID_{50}$/ml method of Reed-Meunch.[15]

Statistical analysis

Single factor analysis of variance (ANOVA) was used to analyze homologous HI titers, macroscopic and histopathological lung scores, IHC and BALF results, log10 transformed nasal swab viral titers, and ADG (JMP 8.0.1, SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$.

Results

Vaccine preparation

The pH1N1 HA gene was inserted into the alphavirus replicon platform and nucleotide sequencing confirmed the correct HA gene sequence had been maintained throughout the cloning process. Western blotting performed on protein lysate confirmed expression of the pandemic HA protein at all the varying HA doses (Figure 1) used in vaccine preparation for the animal study. The HA concentration was determined by pH1N1 HA ELISA and diluted to the specified HA concentration (Table 1).

Antibody titers

Post-vaccination sera were tested for specific antibody response by the homologous HI assay. Hemagglutination inhibition titers were not seen in vaccinated pigs after one dose, but were all positive (≥1:40), except for a single pig in Group 2, at 7

and 14 days post-boost vaccination (data not shown). On the day of challenge, homologous HI titers were significantly higher in groups 2-4 when compared to group 1 (Table 2).

Pathological evaluation

At necropsy, lungs exhibited macroscopic dark purplish-red consolidated lesions located mainly in the cranioventral lobes. Lungs taken from groups 2-4 exhibited significantly lower lesion scores and consolidation than pigs in group 1 (Table 2). There was also a significant reduction in pathological scores in all HA vaccinated groups compared to the placebo vaccinated group (Table 2). The lung sections taken from placebo vaccinated group 1 pigs had approximately 50% of the airways affected by bronchiolar epithelial disruption and peribronchiolar lymphocytic cuffing. The HA vaccinated groups 2-4 demonstrated only occasional affected airways with light cuffing. Swine influenza virus IHC demonstrated that all five lungs taken from placebo vaccinated group 1 pigs were positive for influenza antigen, while only two pigs in total from the HA vaccinated groups 2-4 were positive. Additionally, there were positive trachea IHC samples in all groups (data not shown), but no significant differences were observed between HA vaccinated and placebo vaccinated groups. Positive trachea IHC results correlate with what was previously reported on pathogenesis of pandemic H1N1 in ferrets.[11]

Average daily gain

Groups 3 and 4 exhibited significantly higher ADG over the five day period following challenge than did group 1 (Table 2). Group 2 did exhibit higher ADG but was not significantly higher than group 1 (p=0.08).

Virus isolation

No live influenza virus was detected at one day post-challenge from any nasal swabs (Table 3). On day 2 post challenge live influenza virus was detected in groups 1, 3, and 4, although there were no significant differences between group mean viral titers. On day 3 post-challenge groups 2 and 4 had significantly lower titers than did group 1. On both days 4 and 5 groups 2-4 all exhibited lower titers than group 1. No live virus was detected in nasal swabs from any pigs in group 2 for the duration of the challenge period. Similarly, there was a significant reduction in the number of positive BALF samples between groups (Table 3). By 5 days post- challenge, only a total of three HA vaccinated pigs had detectable live virus in BALF samples, while all five pigs in the placebo vaccinated group had detectable live virus.

Discussion

The recent outbreak of pH1N1 in the human population has highlighted the zoonotic potential of influenza viruses. Even before the current pandemic, there were many reported cases of swine to human transmission of influenza. As such, part of controlling this zoonotic threat is vaccination of swine against swine influenza viruses (SIV). In this study, we demonstrate how rapidly an efficacious swine influenza vaccine based on the alphavirus replicon expression system can be produced in response to an outbreak of a pandemic zoonotic strain.

This study demonstrated the quickness and flexibility with which a vaccine can be produced using the alphavirus replicon expression system. It took less than two months

from the time the pandemic HA sequence was retrieved from GISAID database until pigs were administered the first vaccine dose. Traditional methods for producing influenza vaccines take much longer and are dependent on viral replication in embryonated eggs or on tissue culture cells with subsequent inactivation. In the face of an influenza epidemic, a quick turnaround is important in preventing further transmission and decreasing the zoonotic potential. The alphavirus replicon platform allows for rapid insertion of any influenza HA (or other) gene, making it an attractive influenza vaccine technology due to constant antigenic shift and drift among influenza viruses.

This is the first report of immunization of swine with a recombinant protein produced via an alphavirus replicon expression system. Replicon particle (RP) vaccines produced with this system have recently been utilized to induce protection against SIV.[1,3] The first proof of concept study demonstrated that a replicon particle vaccine (RP) administered to swine was able to induce high antibody HI titers against a human influenza strain. A subsequent study using an RP vaccine expressing the HA protein of a clade IV H3N2 SIV isolate confirmed that influenza HA RP vaccines given to swine are not only able to induce an antibody response, but also provide significant protection against a homologous viral challenge. In contrast to these earlier studies, this study used an alphavirus replicon expression system to produce recombinant HA protein in vitro; however, similar antibody responses and protection from viral challenge was demonstrated.

The results demonstrate that influenza infection of swine with A/California/04/2009 influenza virus is able to induce clinical symptoms and gross lesions comparable to other strains of SIV.[18-20] In contrast with a previous study, several

pigs (primarily in the non-vaccinated group) in this study exhibited clinical signs, mainly coughing and sneezing. This discrepancy may be due to the miniature pig model used in that study.[8] In this study, vaccine administration induced specific antibody titers, reduced macroscopic and histopathologic lung lesions, and reduced viral load in both the nose and lung. Recombinant HA vaccinated pigs also demonstrated a higher average daily gain than placebo vaccinated pigs. These results demonstrate that this recombinant pandemic HA protein is efficacious when used as a vaccine against pH1N1 swine influenza.

Several recent studies have already reported the successful transmission of pandemic H1N1 virus from infected to naïve contact pigs.[2,10] The successful transmission of this virus among pigs and recent confirmation of its presence in the United States demonstrates the need for an efficacious pandemic H1N1 vaccine. This paper shows that vaccination of pigs against pandemic H1N1 can reduce both clinical symptoms and virus shedding in pigs, which may lead to decreased transmission.

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Funding Information

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Figure 1. Western blot confirming recombinant HA protein expression.
Lane 1, Ladder; Lane 2, Vero lysate (negative control);
Lane 3, recombinant HA (28.5µg/ml); Lane 4, recombinant HA (0.57µg/ml);
Lane 5, recombinant HA (0.285µg/ml); Lane 6, recombinant HA (0.19µg/ml)

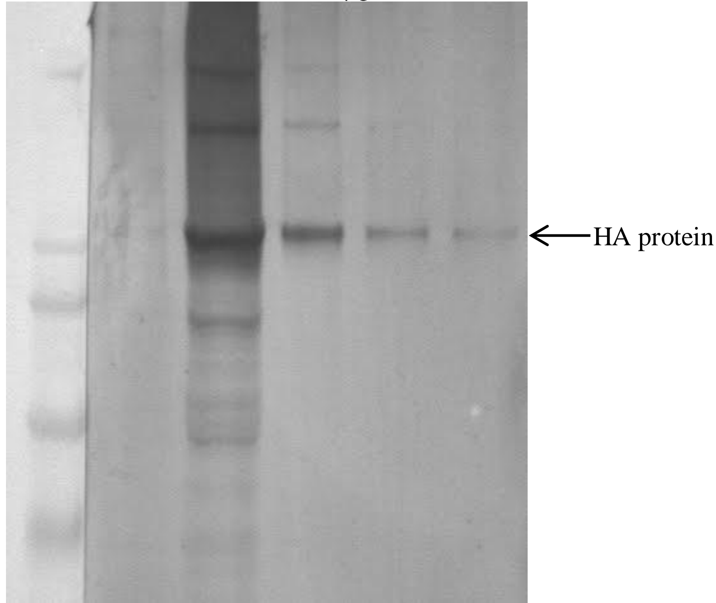

Table 1: Design of pandemic H1N1 recombinant HA vaccine study. Pigs received either placebo vaccine (PBS, Group 1) or varying doses of HA antigen (Groups 2-4). All vaccines were given intramuscularly as 2ml doses on days 0 and 21.

Group	Vaccine	HA concentration/dose
1	Placebo	0.00µg
2	Recombinant HA	1.14µg
3	Recombinant HA	0.57µg
4	Recombinant HA	0.38µg

Table 2. Summary of hemagglutination inhibition (HI) titers, average macroscopic and microscopic lung involvement, immunohistochemistry (IHC), and average daily gain (ADG).

Group	HI Titers[a]	% Pneumonia[b]	Histopathologic Score[c]	Lung IHC[d]	ADG[e]
1	<10	15.6 ± 5.4	1.8 ± 0.1	5/5	1.76 ± 0.2
2	121*	1.4 ± 0.9*	0.8 ± 0.2*	1/5	2.56 ± 0.68
3	184*	0.2 ± 0.2*	0.6 ± 0.2*	0/5	2.64 ± 0.22*
4	106*	1.8 ± 1.1*	0.8 ± 0.2*	1/5	2.45 ± 0.34*

[a]Geometric mean homologous HI titers
[b]Group mean ± standard error
[c]0-3, group mean ± standard error
[d]Number of positive samples per group
[e]ADG post-challenge in pounds, group mean ± standard error
*Values are significantly different from placebo vaccinates (Group 1) within a column at $p<0.05$

Table 3. Summary of live virus isolation from nasal swabs and bronchoalveolar lavage fluid (BALF).

	Nasal Swabs[a]					BALF[b]
Group	1dpc[c]	2dpc	3dpc	4dpc	5dpc	5dpc
1	0	0.85 ± 0.53	2.55 ± 0.66	3.05 ± 0.18	3.05 ± 0.24	5/5
2	0	0	0*	0*	0*	2/5
3	0	1.05 ± 0.07	0.65 ± 0.65	0.9 ± 0.57*	1.0 ± 0.62*	0/5
4	0	0.45 ± 0.45	0.5 ± 0.5*	0.65 ± 0.65*	0.65 ± 0.65*	1/5

[a]Log10 mean virus titers ± standard error in nasal swabs post-challenge
[b]Number of positive BALF samples per group
[c]Days post-challenge (dpc)
* Values are significantly different from placebo vaccinates (Group 1) within a column at $p<0.05$

CHAPTER 4. SAFETY, IMMUNOGENICITY, AND EFFICACY OF AN ALPHAVIRUS REPLICON-BASED SWINE INFLUENZA VIRUS HEMAGGLUTININ VACCINE

A manuscript submitted to *Vaccine*

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Abstract

A single-cycle, propagation-defective replicon particle (RP) vaccine expressing a swine influenza virus hemagglutinin (HA) gene was constructed and evaluated in several different animal studies. Studies done in both the intended host (pigs) and non-host (mice) species demonstrated that the RP vaccine is not shed or spread by vaccinated animals to comingled cohorts, nor does it revert to virulence following vaccination. In addition, vaccinated pigs develop both specific humoral and IFN-γ immune responses, and young pigs are protected against homologous influenza virus challenge.

Introduction

Swine Influenza Virus (SIV) continues to be problematic in the swine industry. Swine influenza virus is characterized by a sudden onset of respiratory illness, and is usually accompanied by anorexia, lethargy, and fever. In addition to the clinical complications associated with SIV in production animals, there have been several published reports implicating swine in the transfer of influenza viruses to humans.[24,32,39] Most recently, the 2009 pandemic H1N1 influenza virus was shown to have components of swine origin.[9]

Our group has reported the rapid development of a swine vaccine against the pandemic H1N1 virus based on the alphavirus replicon system.[36] In addition to the possibility of zoonotic transfer, swine influenza viruses within the swine population continue to evolve at a rapid pace. Until 1998, swine influenza in the United States was caused almost exclusively by classical H1N1,[7] originally isolated in 1930.[33] However, in 1998 both double and triple reassortant H3N2 viruses emerged.[38,41,42] Since then, there have been many influenza reassortment events that have led to the emergence of new subtypes and clusters.[5,11,12,19,30,37,40] Commercially available SIV vaccines often do not protect against new and emerging virus subtypes/clusters and must be periodically updated to match currently circulating strains. As such, novel swine influenza vaccines that are safe, effective, and can be rapidly altered to antigenically match an emerging strain should be considered as alternatives to traditional swine influenza vaccines. The United States Department of Agriculture Center for Veterinary Biologics (USDA CVB) has guidelines on the design of such safety studies for modified live vaccines (Veterinary Services Memorandum 800.201), but to date, has no specific guidance on shed spread or reversion to virulence studies for recombinant replication-incompetent vaccines. Thus, the studies included in this paper represent novel study designs and results that have been approved by the USDA CVB specifically for this replication-incompetent alphavirus-based replicon particle (RP) SIV vaccine.

An alphavirus replicon vector system has been derived from the attenuated TC-83 strain of Venezuelan equine encephalitis virus (VEEV).[14] The ~11.4kb VEEV positive-sense genome contains two open reading frames (ORFs). The 5' ORF encodes four nonstructural proteins (nsp1-4) and the 3' ORF encodes the virus structural proteins

(capsid and glycoproteins (E3, E2, 6K and E1)).[35,27] The nonstructural proteins are translated from the positive-sense genomic RNA and function to transcribe full-length negative-sense RNA. This negative-sense RNA is a template for both additional genomic RNA as well as 26S subgenomic mRNA. The 26S promoter is located between the two ORFs on the negative-sense RNA and is recognized by the nonstructural proteins for transcription of a subgenomic mRNA, from which the structural proteins are translated. This 26S mRNA is produced in 10-fold molar excess when compared to genomic RNA.[35] Foreign genes of interest can be inserted in the place of VEEV structural genes in a cDNA clone generating a self-replicating RNA (replicon) capable of expressing the foreign gene when introduced into cells. The self-amplifying replicon RNA directs the translation of large amounts of protein in transfected cells, reaching levels as high as 15-20% of total cell protein.[26] This replicon RNA does not contain any of the VEEV structural genes, so the RNA is propagation-defective. The replicon RNA can also be packaged into a replicon particle (RP) by supplying the structural genes *in trans* in the form of capsid and glycoprotein helper RNAs.[18,26] When the helper and replicon RNAs are combined and cotransfected into cells, the replicon RNA is efficiently packaged into single-cycle, propagation-defective RP.

Early production of RP were hampered by recombination events that resulted in the generation of replication-competent virus (RCV)[27]; first generation helper RNAs encoded the capsid and glycoprotein genes on the same RNA molecule, and thus only required one recombination event to create RCV.[2,10,26] The probability of this event occurring was greatly reduced by separating the helpers onto two separate RNAs ("split helper" system). This bipartite or split conformation greatly reduced the occurrence of

RCV, as separation of the helper RNAs requires two independent recombination events to occur for generation of RCV.[26,34] These initial versions of the helper RNAs were designed to contain a 26S promoter downstream of the 5' untranslated region. However, recent studies have demonstrated that the 26S promoter is not required for functional helper RNAs.[16] Removal of the 26S promoter results in helper RNAs that are not independent transcriptional units, and further reduces the possibility of functional recombinations between the replicon and helper RNAs. In addition to the removal of the 26S promoter, a stop codon has been introduced at the 3' end of the capsid gene in place of the chymotrypsin-like cleavage site.[16] This mutation negates the cleavage activity of the capsid protein, adding another safeguard against functional recombination. Thus, helper RNAs lacking 26S promoters and with an engineered capsid stop codon further reduce the probability of functional recombination than the standard split helper RNA system.[16]

Alphavirus RP vaccines have been tested in multiple animal studies using multiple species (including humans) for more than 20 years.[3,8,15,27] However, formal safety studies have not been conducted in swine previously. We have used the alphavirus replicon system to produce an H3 SIV RP vaccine and we report studies performed evaluating the potential for this vaccine to shed, spread, and revert to virulence in both the intended host (pigs) and non-host (mice) species. Immunogenicity and efficacy were also evaluated in pigs of different ages.

Material and Methods

Replicon particle vaccine

The HA gene was PCR amplified from a cluster 4 H3N2 SIV isolate and cloned into the VEEV RP vector system using previously published methods.[4]

Pig shed spread and reversion to virulence study

Twenty six-week old caesarean-derived, colostrum-deprived (CDCD) pigs (12 gilts and 8 barrows) were obtained from Struve Labs (Manning, IA). All pigs were confirmed negative for antibodies to porcine reproductive and respiratory syndrome virus (PRRSV), SIV H1N1 and SIV H3N2 using commercial ELISA assays (IDEXX). The pigs were randomly assigned to HA RP vaccinated or placebo groups. Upon arrival at the study facility, pigs were separated into five different pens located within the same room. Each pen contained two H3 RP vaccinated pigs and two placebo pigs. These two groups were comingled for the duration of the study, except for the 24 hours immediately following vaccination to prevent physical transmission of the H3 RP vaccine to placebo pigs. The H3 RP vaccine was administered both intravenously (IV) in the right jugular vein and intramuscularly (IM) on the right side of the neck, both in 3 ml doses containing $1x10^{10}$ H3 RP, for a total of $2x10^{10}$ H3 RP. The placebo vaccine containing only the vaccine diluent was administered in identical dose volumes and injection sites. Both vaccines were administered by personnel blinded to vaccine composition to avoid potential bias regarding vaccine reactions. Pigs were observed daily for 14 days post-vaccination for any vaccine-related adverse effects. Serum, nasal swabs and rectal swabs were collected on study days -1, 0, 3, 7, 10, and 14. Nasal and rectal swabs were placed into 15 ml conical tubes containing 1ml minimum essential media (MEM) (Invitrogen Life

Technologies) + 1% antibiotic/antimycotic (Invitrogen Life Technologies). Samples were held at -80°C until removal for further processing. Tissues collected at necropsy included injection site (right neck musculature), tonsil, spleen, heart, lung, right retropharyngeal lymph node, liver, intestine, brain, and kidney. Tissues were placed in whirl-pak bags at necropsy and held at -80°C until removal for further processing. Samples collected throughout the study were assayed by the RT-PCR and CPE assays. All pigs were housed and treated in accordance with IACUC approved guidelines.

Mouse shed spread and reversion to virulence study

Twenty six-week old BALB/c female mice were obtained from Charles River Laboratories (Portage, MI). The twenty mice were divided equally among five cages, with each cage containing two mice injected with H3RP vaccine and two mice injected with placebo vaccine. Mice were identified within a cage by unique individual ear notches. The two groups were comingled for the duration of the study, except for the 24 hours immediately following vaccination on study days 0 and 14 to prevent physical transmission of the H3 RP vaccine to the placebo mice. The H3 RP vaccine was delivered intraperitoneally in 200µl doses containing $3x10^7$ SIV RP. The placebo vaccine was administered in identical dose volumes and injection sites. Both vaccines were administered by personnel blinded to vaccine composition to avoid potential bias regarding vaccine reactions. Fecal pellets were collected from each cage at multiple time points throughout the study. Blood was collected post-euthanasia via cardiac puncture. Tissues collected at necropsy included brain, liver, heart, kidney, spleen, lungs, and intestine. A portion of each tissue was placed into individual microcentrifuge tubes, and a portion of the tissue samples were also fixed in 10% buffered formalin for

histopathological analysis. All samples collected throughout the study and at necropsy were held at -80°C freezer until removal for further processing. Samples collected throughout the study were assayed by the RT-PCR and CPE assays. All mice were housed and treated in accordance with IACUC approved guidelines.

H3 RP vaccine immunogenicity study

Eighteen first parity gilts were obtained from Wilson's Prairie View Farm, Inc. (Burlington, WI). All gilts were confirmed negative for antibodies to PRRSV and SIV. Gilts were randomized equally into three treatment groups. Group 1 received H3 RP, group 2 received PRRSV RP, and group 3 received no treatment. Groups 1 and 2 received respective RP vaccines at a dose of $2x10^9$ IM on study days 0 and 21. Serum and peripheral blood mononuclear cells (PBMC) were collected at 15 days post-booster vaccination for homologous HI testing and IFN-γ ELISPOT, respectively.

H3 RP vaccine efficacy study

Fifty three-week old pigs (27 gilts and 23 barrows) were obtained from Wilson's Prairie View Farm, Inc. (Burlington, WI). All pigs were confirmed negative for antibodies to PRRSV and influenza nucleoprotein (ELISA), and SIV H1N1 and SIV H3N2 (HI). In addition, all pigs were negative for antibodies against the homologous cluster 4 H3N2 SIV strain used in the study as determined by HI assay. Pigs were randomized by litter to treatment groups and housing units. Treatment groups consisted of 20 H3 RP vaccinates, 20 placebo vaccinates, and 10 strict negative controls. All the pigs were housed in the same room in 2 separate pens, each pen containing 10 H3 RP vaccinates, 10 placebo vaccinates, and 5 strict controls. H3 RP vaccinated pigs received a 2ml dose containing $1x10^8$ RP administered IM on study days 0 and 21. The placebo

vaccine containing only the vaccine diluent was administered in identical dose volumes and injection sites. The strict controls were not vaccinated at any time throughout the study. Both vaccines were administered by personnel blinded to vaccine composition to avoid potential bias regarding vaccine reactions and clinical observations. Pigs were observed daily throughout the study for vaccine-related adverse effects. On study day 56 (35 days post-booster vaccination) the H3 RP and placebo vaccinated pigs were challenged intratracheally with the homologous (to the H3 RP vaccine) cluster 4 H3N2 SIV strain at a dose of $1x10^7$ $TCID_{50}$ in a 10ml volume. The strict controls were not challenged and were moved to a separate room prior to challenge. Body temperatures were collected from each pig pre- and post-challenge using injectable RFID chips and a hand-held scanner (Destron Fearing). Nasal swabs were collected daily beginning on the day of challenge until necropsy for live virus titration. Pigs were euthanized and necropsied 4 days post-challenge. At necropsy, each lung lobe affected by pneumonia was visually estimated by a blinded board-certified veterinary pathologist, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume.[13] A portion of each lung lobe was collected in 10% buffered formalin for histopathological analysis and scored as described previously.[30] Bronchoalveolar lavage fluid (BALF) samples were collected from each pig at necropsy for live virus titration.

RNA extraction and RT-PCR

RNA was extracted from serum samples using the Qiagen QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. RNA was extracted from feces and all tissues using the Qiagen QIAshredder and the Qiagen RNeasy Mini Kit according

to the manufacturer's instructions. Briefly, feces and tissue samples were mixed with an appropriate amount of Buffer RLT and homogenized using ground glass tissue grinders. Homogenates were run through individual QIAshredder columns, and RNA was extracted as per the RNeasy Mini Kit protocol. RT-PCR was performed using the Qiagen OneStep RT-PCR Kit with 5 µl of RNA template. Primers were designed to amplify a 300 base pair segment of the SIV H3 gene contained in the SIV H3 RP vaccine. Primer sequences were SIV H3-RP-For (5'GCATATTCGGCGCAATAGCAGGTT-3') and SIV H3-RP-Rev (5'-GCAACAAGAAGCTCCGCGTTGTAA-3'). Swine influenza virus H3N2 HA RNA and nuclease-free water were included in each PCR run as positive and negative controls, respectively. Cycle conditions were 58°C for 30 minutes, 95°C for 15 minutes, 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, and a final hold at 4°C. PCR products were then separated by electrophoresis on a 2% agarose gel, visualized and photographed using ultraviolet light on a BioRad GelDoc. The limit of detection for this assay was determined by calculating the number of PCR copies visually detected by UV illumination of serially diluted *in vitro* transcripts of the H3 replicon.

Cytopathic effect (CPE) assay

Samples (including serum, swabs, and tissues) obtained throughout the studies were evaluated for the presence of RCV by the CPE assay. Tissue samples were prepared by adding 2 parts MEM + 1% antibiotic/antimycotic to 1 part (v/w) of respective tissue and homogenizing with a ground glass tissue grinder. The homogenate was centrifuged, and the resulting supernatant was used for the CPE assay. No further processing was done on serum before the CPE assay. The following CPE assay is

adapted from the published CPE assay method.[17] Each serum and tissue sample collected throughout the study was used to inoculate one well of a 48 well plate containing a monolayer of Vero cells. After 1 hour incubation at 37°C/5% CO_2, samples were decanted, all wells were washed three times with PBS, and fresh MEM placed on the cells. After 24 hours incubation, media supernatants were transferred to a fresh Vero cell 48 well plate and incubated for 1 hour. After incubation, media was decanted, cells washed three times with PBS, and fresh MEM added to cells. After an additional 72 hours of incubation, the cells were examined microscopically for CPE. Cells were then fixed and stained using a 10% formalin solution with 0.25% crystal violet. This method also allowed for macroscopic evaluation of the cells for CPE. MEM was used as a negative control and green fluorescent protein (GFP) RP was used as a positive control to ensure RP infection of Vero cells. The CPE assay has a limit of detection of 1 PFU of replication-competent TC-83 VEE virus in the presence of $1x10^{10}$ replicon particles (Kamrud unpublished results).

IFN-γ ELISPOT

The IFN-γ ELISPOT was performed using a modification of previously described methods.[43] Briefly, Millipore Multi-Screen filter 96 well plates were pre-wet with 70% ethanol, washed with PBS, coated with purified mouse anti-swine IFN-γ (BD Biosciences), and incubated overnight at 4°C. Following incubation, plates were washed with RPMI media (Sigma-Aldrich) and blocked for 1 hour at room temperature with RPMI containing fetal bovine serum and antibiotics (complete RPMI). After blocking, the media was decanted and each PBMC sample was plated in replicates at a concentration of 2e5 PBMC/well. PBMC plus complete RPMI was used as the

unstimulated negative control and PBMC plus PHA-P at 10µg/ml was used as the positive control. PBMC were plated in duplicates and then stimulated with whole virus H3N2 influenza virus supernatant at a titer of $1x10^6$ $TCID_{50}$. The plates were incubated with the stimulating antigens for 18-22 hours at 37°C. After incubation, the plates were washed with 1x KPL wash solution. Bioatinylated mouse anti-swine IFN-γ (BD Biosciences) was added to each well and incubated for 1 hour at 37°C. After washing, alkaline phosphate labeled streptavidin (Bio-Rad) was plated and incubated for 1 hour at 37°C. Plates were developed for 3 to 10 minutes using an alkaline phosphatase substrate kit (Bio-Rad). Positive spots were enumerated using a Zeiss ELISPOT reader system (Zellnet Consulting Inc.) The number of IFN-γ producing PBMC was determined by subtracting the number of spots from the wells stimulated with antigen from the wells with no stimulation. This number was then normalized to spot forming cells/$1x10^6$ PBMC.

Live virus titration

Nasal swab and BALF samples were subsequently thawed and vortexed. The swabs were removed and tubes were centrifuged to pellet cell debris. One milliliter of media was removed without disturbing the pellet and transferred to a sterile microcentrifuge tube for live virus titration. Serial 10-fold dilutions of the sample were performed in MEM supplemented with TPCK-Trypsin (Thermo Scientific) and antibiotic/antimycotic. 100µl of each dilution was transferred to confluent MDCK cells in 96 well plates and incubated for 3 days at 37°C/5% CO_2. Following incubation, cells were fixed with a 70%/30% acetone/methanol solution and washed with PBST. Infected cells were visualized by IFA using a mouse monoclonal antibody specific for the

Influenza A nucleoprotein with high specificity for N2/N3 type Flu A (Millipore) and Alexa Fluor 488 donkey anti-mouse IgG (H+L) (Invitrogen Life Technologies). Plates were observed using an inverted fluorescent microscope to count infected cells. Titers were determined using the Reed-Muench equation.[29]

Statistical analysis

Analysis of variance (ANOVA) was used to analyse inverse homologous HI titers, ELISPOT counts, and log10 transformed live virus titrations. The non-parametric Wilcoxin rank sum test was used for gross and histopathological lung score analysis. Analyses were performed using the JMP software (SAS, Cary, NC). Statistical significance was set at $p<0.05$.

Results

Shed spread/reversion to virulence studies

All of the samples collected in both the pig and mice shed spread studies, including tissues, serum, nasal and rectal swabs, and fecal pellets, were tested by an H3-specific RT-PCR assay to determine if the H3 RP vaccine demonstrated any capacity for shed or spread. No H3-specific RNA was detected in any of the samples taken from H3 RP or placebo vaccinated animals. The limit of detection for the RT-PCR assay was determined to be $1.5x10^3$ copies per reaction. Homologous HI tests were conducted on pig serum collected at necropsy (14 days post-vaccination) to demonstrate that the RP vaccine was indeed functional. All of the pigs receiving the H3 RP vaccine developed positive HI titers ranging from 20-80 (GMT = 57, data not shown), while all of the pigs receiving placebo vaccine were negative at the conclusion of the study.

Histopathological analysis demonstrated that there were no significant lesions identified in either H3 RP or placebo vaccinated mice, and none of the non-specific lesions were consistent with either VEEV or SIV infection. Samples collected in both the pig and mice shed spread studies were negative for any RCV following two blind passages on Vero cells by both microscopic and macroscopic evaluation.

Immunogenicity study

When the homologous H3N2 virus was used as the stimulating antigen in the ELISPOT assay, there was a specific IFN-γ response in gilts that received the H3 RP vaccine (Table 1). In addition, all gilts receiving the H3 RP vaccine demonstrated high homologous HI titers at 15 days post-boost (Table 1).

Efficacy study

The homologous H3N2 HI serum antibody titers are shown in Table 2. At three weeks post-prime vaccination and prior to receiving a booster injection, 19 of the 20 H3 RP vaccinated pigs had already developed positive HI titers ranging from 20 to 160 (data not shown). A significant increase following booster vaccination was observed which was maintained until the day of challenge at 5 weeks post-boost. Sera from placebo vaccinated pigs and the strict controls were negative for HI antibody throughout the study.

Body temperatures of pigs collected post-challenge are summarized in Table 3. At 1 day post-challenge, the H3 RP vaccinated pigs had significantly lower body temperatures than placebo vaccinated pigs. The febrile response peaked at an average of 40°C at 1 day post-challenge in the placebo vaccinated group after which all body temperatures began to decline to baseline levels. At day 1 post-challenge, 50% of the

placebo vaccinated pigs developed fevers (≥40°C) while only 20% of H3 RP vaccinated pigs developed fevers.

Nasal shedding and lung viral titers are shown in Table 2. The H3 RP vaccine prevented detectable nasal shedding at all days post-challenge, while viral shedding was detected in 18 of 20 placebo vaccinated pigs by day 4 post-challenge. In addition, no live virus was detected in the BALF samples from the H3 RP vaccinated pigs, while all pigs receiving placebo vaccine had detectable virus recovered from the BALF samples.

Macroscopic evaluation of individual lungs at necropsy exhibited pneumonic lesions typical of SIV with purplish-red areas of consolidation. H3 RP vaccinated pigs exhibited statistically significant lower lung lesion scores than the placebo vaccinated animals (Table 3). The macroscopic lung scores were similar in both non-challenged controls and the H3 RP vaccinated pigs. Histopathological analysis of lungs correlated with the macroscopic results. Ninety percent of placebo vaccinated pigs exhibited epithelial disruption with interstitial pneumonia in at least 30% of the upper airways (score ≥ 2). In contrast, only 20% of the H3 RP vaccinated pigs exhibited a few disrupted airways (score ≤ 1).

Discussion

The capacity for shed, spread and reversion to virulence of an SIV H3 RP vaccine was evaluated in both the intended host (pigs) and non-host (mice) species in a study design that was approved by USDA CVB. The rational for using a 200-fold higher dose in the safety studies compared to the efficacy study was to conclusively demonstrate the safety of the vaccine even when administered at a dose orders of magnitude higher than

the intended dose. An H3-specific RT-PCR assay was utilized to detect shedding of the RP vaccine. If recombination event(s) were to occur resulting in replication-competent virus (RCV), the H3 gene would likely be partially or completely replaced by the structural capsid and glycoprotein genes. However, the purpose of the RT-PCR was not to necessarily detect RCV, but instead to monitor various tissue and secretory samples for shedding of the vaccine RNA. Even at this high dosage level, no replicon-specific RNA was detected in nasal and rectal swabs, serum, or tissues at any time point of sample collection. These results demonstrate that the H3 RP vaccine is not shed from vaccinated animals nor spread to non-vaccinated cohorts or into the environment. The assay used to demonstrate the absence of RCV has been shown to be very sensitive (detection of 1 PFU in $1x10^{10}$ RP, data not shown). All of the samples tested were negative for RCV following two blind passages in cell culture, indicating a lack of reversion to virulence to the parental VEEV strain. In addition, all of the H3 RP vaccinated animals were HI positive by 14 days post-vaccination while none of the placebo vaccinated pigs had positive serum HI titers. These results indicate that the vaccine was functional and further demonstrate the lack of shed and spread of the H3 RP vaccine. We believe that these results support the inherent safety of the replicon system.

This is the first report evaluating the safety profile of RP vaccines in swine. However, extensive safety and biodistribution studies have been completed in non-swine models. Several VEEV RP toxicology studies performed in rabbits indicate no adverse reactions following IM and SC injections.[15,28] Kowalski et al demonstrated that intracranial (IC) injection of VEEV RP resulted in only transient pathology (1-2 days) and weight loss (1 day) with a rapid return to pre-injection status.[20] Vaccine RNA was

detectable in brain tissue for only 5-8 days following IC injection, but no RNA was detected when the RP was administered IV or IM, suggesting that it does not cross the blood-brain barrier after systemic administration. The neurovirulence of both Sindbis and Semliki Forest virus (SFV) RP expressing LacZ have been evaluated following IC inoculation. Beta-galactosidase activity was detectable in brains for 14-28 days, but no morbidity, neuropathology, or loss of motor skills was observed in either study, indicating a lack of reversion to the parental virus strains.[1,21] A biodistribution study done with the same SFV RP system demonstrated that following IC injection there was no evidence of RP RNA colocalization to either the liver or the brain after 15 days, and no abnormalities were observed during histopathological examination.[22] The results from these non-swine studies correlate with the host (pig) and non-host (mice) species results presented here, specifically that no reversion to virulence was noted *in vivo*.

As demonstrated by the results presented here, the current features of the VEEV RP system result in a safe vaccine that is not shed or spread nor does it revert to virulence. However, if in such a rare circumstance the perfect pair of recombinations did occur in the exact order and positions needed,[16] the resulting RCV would theoretically be no more virulent than the parent VEEV strain TC-83. TC-83 has been used extensively to vaccinate military personnel and lab workers against VEEV infection. A retrospective study of hundreds of humans who received the vaccine from 1976 to 1990 indicated that TC-83 vaccine caused some transient reactions, but no serious sequelae were reported.[25] This strongly suggests that if multiple improbable functional recombination events were to occur, the outcome would not have serious public health consequences.

The current studies also demonstrate that the VEEV RP system is an immunogenic and efficacious vector for swine vaccines. Other studies have previously demonstrated immunogenicity and efficacy of RP expressing HA antigens.[4,8,15,26,31] However, the studies presented here are the first to report a specific CMI response in pigs and to demonstrate formal efficacy in a large controlled study. Following H3 RP vaccination, both a humoral and specific IFN-γ response was observed in pregnant gilts. In the young pig efficacy study, IFN-γ levels were not examined, but robust HI titers were demonstrated out to 5 weeks post-booster vaccination. Efficacy was demonstrated by preventing or reducing nasal shedding, viral replication in the lungs, body temperature, and lungs lesions when compared to placebo vaccinated animals. In addition, only H3 RP vaccinated pigs in the efficacy study developed positive HI titers, while all of the controls and placebo pigs remained seronegative, further demonstrating a lack of shed and spread of the vaccine. Thus, the H3 RP vaccine has been shown to be a safe and effective alternative to traditional vaccines used to control SIV.

Swine vaccines based on the alphavirus RP system offer many advantages over other traditional commercial vaccines. First, the RP is propagation-defective. This feature alleviates concerns regarding reversion to virulence in vaccinated animals. The molecular safety features of the current RP vector system ensure that the risk of reversion to virulence is negligible, and the present studies confirm that safety in both the intended host (pigs) and non-host (mice) species. Second, the H3 RP vaccine is able to differentiate infected from vaccinated animals (DIVA). Current killed vaccines contain inactivated whole viruses, and as such induce immune responses to multiple flu antigens not necessarily related to a protective response. The H3 RP vaccine contains only the HA

gene so it can be used in conjunction with the current diagnostic NP-specific influenza detection test[6] to easily determine the infection status of an animal or herd. Third, because it has been shown that vaccination in the presence of pre-existing immunity to RP has no or only minimal effect on inducing robust host immune response, the same RP vector can be used for multiple vaccinations of the same animal with no decrease observed in vaccine efficacy.[23,26] This feature also allows for the same RP vector to be used as vaccines against different diseases. Finally, the RP vector system can be utilized to include protective genes from most any pathogen. The requirement for high-containment laboratories to carry out research or vaccine production is not an issue with the RP system because genes of interest can be *de novo* synthesized and engineered directly into a replicon vector without the need for growth of the actual pathogen. Furthermore, because the vector is easily manipulated at a molecular level, vaccines can be produced quickly in response to emerging infectious diseases.[36]

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Table 1. IFN-γ ELISPOT and homologous hemagglutination inhibition (HI) results at 15 days post-boost vaccination.

Group	IFN-γ ELISPOT[a]	HI Titers[b]
H3 RP	203.3 ± 89.4	403.2 ± 86.8
PRRSV RP	10.8 ± 7.1	0.0 ± 0.0
Control	10.4 ± 8.2	0.0 ± 0.0

[a]Number of IFN-γ secreting-cells/$1x10^6$ PBMC, group mean ± SEM

[b]Inverse geometric mean titer (GMT), group mean ± SEM

Table 2. Serum homologous hemagglutination inhibition (HI) antibody titers and influenza virus titers in nasal swabs and lungs of vaccinated and control pigs in efficacy study.

Group	Homologous HI Titers[a]				Log_{10} Virus Titers[b]		
	Pre-Vac	0dpb	7dpb	35dpb	NS 3dpc	NS 4dpc	BALF
Placebo/Ch	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.3	2.8 ± 0.3	4.2 ± 0.2
H3 RP/Ch	0.0 ± 0.0	33.3 ± 8.2	≥320.0 ± 40.0	≥234.3 ± 48.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control/NoCh	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Ch: Challenged; NoCh: Not Challenged; dpb: days post-booster vaccination; NS: nasal swabs; dpc: days post-challenge

[a]Inverse geometric mean titer (GMT), group mean ± SEM

[b]SIV $TCID_{50}$/ml log_{10} titers. No live virus detected at 0, 1, and 2 days post-challenge in any group. Group mean ± SEM

Table 3. Body temperature, gross and microscopic lung lesions of vaccinated and control pigs in efficacy study.

Group	Body Temperature (°C)[1]				Gross Lung Scores[2]	Histopathological Lung Scores[3]
	1dpc	2dpc	3dpc	4dpc		
Placebo/Ch	40.0 ± 0.1[a]	38.4 ± 0.1[a]	38.4 ± 0.1[a]	38.8 ± 0.1[a]	18.7 ± 1.8[a]	2.3 ± 0.1[a]
H3 RP/Ch	39.1 ± 0.2[b]	38.1 ± 0.1[a]	38.3 ± 0.2[a]	38.3 ± 0.2[b]	2.6 ± 1.3[b]	0.2 ± 0.1[b]
Control/NoCh	38.4 ± 0.2[c]	38.3 ± 0.2[a]	38.6 ± 0.2[a]	38.6 ± 0.2[ab]	1.1 ± 0.5[b]	0.0 ± 0.0[b]

Ch: Challenged; NoCh: Not Challenged; dpc: days post-challenge

[1]Group mean ± SEM

[2]Total percentage (%) of lung affected by macroscopic pneumonic lesions [31], group mean ± SEM

[3]Scores are based on a 0-3 scale [13], group mean ± SEM

[a,b,c]Groups with different letter superscripts are significantly different at $p \leq 0.05$

CHAPTER 5. VARYING LEVELS OF PROTECTION AGAINST PANDEMIC H1N1 IN PIGS FOLLOWING HEMAGGLUTININ AND NUCLEOPROTEIN REPLICON PARTICLE VACCINATION

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Abstract

The emergence of the pandemic H1N1 (pH1N1) influenza virus in 2009 highlights the zoonotic potential of influenza viruses and also the need for vaccines capable of eliciting heterosubtypic protection. In these studies single-cycle, propagation-defective replicon particle (RP) vaccines expressing influenza hemagglutinin (HA) and nucleoprotein (NP) genes were constructed and efficacy was evaluated in homologous and heterologous animal challenge studies with the pH1N1 influenza virus. Homologous HA RP vaccination protected pigs against pH1N1 challenge. An RP vaccine expressing an H3N2-derived NP gene was able to decrease nasal shedding and viral load following heterosubtypic pH1N1 challenge in pigs. These studies indicate that although homologous vaccination remains the most effective means of preventing pH1N1 influenza infection, other vaccine alternatives do offer a level of heterosubtypic protection and should continue to be evaluated for their ability to provide broader protection.

Introduction

The recent emergence of the pandemic H1N1 (pH1N1) influenza strain in the global human and animal populations highlights the zoonotic potential of influenza viruses. This 2009 pH1N1 influenza virus has been shown to contain genes that are of swine origin.[8] In addition to the pandemic strain, there have been several published reports of zoonotic transfer of influenza viruses from pigs to humans.[16,22,29] In addition, many subtypes and clusters of swine influenza virus (SIV) currently co-circulate among the United States swine population.[4,11,20,28,33] Thus, vaccination against SIV represents the best option for decreasing clinical complications in swine as well as decreasing opportunities for zoonotic spread from swine to humans.

Studies have shown that homologous vaccination against the pH1N1 virus represents the most efficacious vaccine option, although there appears to be some cross-protection from non-homologous vaccines that contain influenza strains from the same phylogenetic cluster.[30,31] Recently, our group reported the rapid development of a recombinant hemagglutinin (HA) protein vaccine for swine against pH1N1 using the alphavirus replicon system.[26] Because only the HA gene sequence is required to begin vaccine production using the alphavirus replicon platform system and not the actual virus isolate, replicon-based vaccines can be produced more rapidly than traditional inactivated vaccines in response to emerging viruses. The replicon system has been used to express genes from numerous pathogens in addition to influenza virus, including simian immunodeficiency virus, Norwalk virus, Ebola virus, Lassa virus, and equine arteritis virus.[18]

The alphavirus replicon system has been used to produce influenza HA vaccines for evaluation in several different species, including poultry, humans, and swine.[2,7,14,21] However, the recent pH1N1 outbreak has increased interest in producing universal influenza vaccines that offer heterosubtypic protection. The main area of research in developing universal influenza vaccines has focused on the use of conserved antigens, such as nucleoprotein (NP) or M2 protein, to elicit broadly reactive immune responses. The NP is very highly conserved, with ~89% homology among 955 sequences from wild and domestic birds, humans, swine and equine.[37] Traditionally, it was thought that the immune response to NP was dictated mainly by cytotoxic $CD8^+$ T cells[36] and cytokine-secreting $CD4^+$ T cells.[25] However, there is evidence that both NP-specific antibodies and cell-mediated immunity (CMI) play important roles in protection from influenza challenge.[3,5,17] Thus, a vaccine that is capable of inducing both an anti-NP antibody and a cell-mediated immune response is needed to provide sufficient heterosubtypic immunity. Replicon particle (RP) vaccines have been shown to induce robust antibody responses in swine,[2,7] and to increase interferon-gamma (IFN-γ) producing cells[14] and $CD8^+$ T cells.[27]

In the present studies we utilize the alphavirus replicon system to produce RP vaccines expressing influenza HA and NP genes. These vaccines were evaluated in pigs and tested for efficacy following virulent A/California/04/2009 pH1N1 challenge.

Materials and Methods

Replicon particle vaccine production

The pandemic H1N1 (pH1N1) A/California/04/2009 influenza virus hemagglutinin (HA) nucleotide sequence was retrieved from the Global Initiative on Sharing Avian Influenza Data (GISAID) database. The nucleoprotein (NP) nucleotide sequence was determined from a cluster 4 H3N2 SIV field isolate. These sequences were optimized for expression and *de novo* synthesized (DNA2.0). The genes were synthesized with unique 5' and 3' restriction sites which allowed insertion into the alphavirus replicon platform and an optimized construct was selected as previously described.[15] RNA transcripts were produced *in vitro* and, along with TC-83 structural genes, mixed with Vero cells in electroporation cuvettes, pulsed, and incubated overnight prior to harvest as previously described.[15] The resulting RP titers were determined using replicon-specific immunofluorescence assays.

Study 1: Homologous pH1N1 HA RP efficacy study

Ten three-week old pigs were obtained from Wilson's prairie View Farms (Burlington, WI). All pigs were confirmed negative for antibodies to porcine reproductive and respiratory syndrome virus (PRRSV) and SIV by commercial ELISAs (IDEXX). In addition, all pigs were confirmed to be negative for pH1N1 antibodies by the hemagglutination inhibition (HI) assay. The pigs were randomly and equally assigned to one of two treatment groups: HA RP vaccine or placebo vaccine (vaccine diluent only). The HA RP was administered intramuscularly at a $1x10^8$ RP/2ml dose. The first dose of vaccine was administered to pigs on study day 0 and booster vaccination given on study day 21. Sera samples were collected throughout the study to evaluate

serum antibody HI titers. On study day 47 all pigs were challenged intratracheally with virulent A/California/04/2009 pH1N1 influenza virus at a dose of $2x10^5$ $TCID_{50}$. Nasal swabs were collected daily following challenge by swabbing each naris with a polyester tipped swab and placing in a 15ml conical tube containing Minimum Essential Media (MEM) plus antibiotics/antimycotic (Invitrogen Life Technologies). All pigs were euthanized and necropsied 5 days post-challenge. Each lung lobe was grossly examined by a board-certified veterinary pathologist blinded to the experimental groups, and a total percentage of each lung affected by pneumonia was calculated based on weighted proportions for each lobe to the total lung volume.[12] A portion of each lung lobe was collected in 10% buffered formalin for histopathological analysis and scored as described previously.[20] Each lung lobe was also evaluated for the presence of SIV antigen by SIV-specific immunohistochemistry (IHC) staining. Bronchoalveolar lavage fluid (BALF) samples were collected from each pig at necropsy by pipetting MEM plus antibiotics/antimycotic into the trachea followed by gentle massaging of the lobes and subsequent recovery of the media. Nasal swabs and BALF samples were frozen at -80°C until use in the live virus titration assay. All pigs were weighed immediately prior to challenge and again post-mortem at necropsy for determination of average daily gain (ADG). All animal studies were performed using protocols approved by the Iowa State University Institutional Animal Care and Use Committee.

Study 2: NP RP efficacy study

Twenty three-week old pigs were obtained from Wilson's Prairie View Farms (Burlington, WI). All pigs were confirmed negative for antibodies to PRRSV and SIV as described above. The pigs were randomly and equally assigned to one of two treatment

groups: NP RP vaccine or placebo vaccine (RP expressing an irrelevant gene of interest, PRRSV Membrane protein). Both RP vaccines were administered intramuscularly at a dose of $1.2x10^9$ RP/2ml. The first dose of vaccine was administered on study day 0 and booster vaccination given on study day 21. Sera samples were collected throughout the study to evaluate NP ELISA antibody titers. On the day of boost and day of challenge whole blood samples were collected for the isolation of peripheral blood mononuclear cells (PBMC) for subsequent use in the IFN-γ assay. On study day 42 all 20 pigs were challenged transtracheally with virulent A/California/04/2009 pH1N1 influenza virus at a dose of $2x10^5$ $TCID_{50}$. All pigs were euthanized and necropsied 5 days post-challenge. Post-challenge samples were collected as described for Study 1.

Live virus titration

Nasal swab and BALF samples were subsequently thawed and vortexed. The swabs were removed and tubes were centrifuged to pellet cell debris. One milliliter of media was transferred to a sterile microcentrifuge tube for use in the live virus titration assay. Serial 10-fold dilutions of the samples were performed in MEM supplemented with TPCK-Trypsin (Thermo Scientific) and antibiotics/antimycotic. Each dilution was transferred to confluent MDCK cells in 96 well plates and incubated for 3 days at 37°C/5% CO_2. Following incubation, cells were fixed with a 70%/30% acetone/methanol solution and washed with PBS. Infected cells were visualized by IFA using a mouse monoclonal antibody specific for the Influenza A nucleoprotein with high specificity for N1 type Flu A (Millipore) and Alexa Fluor 488 donkey anti-mouse IgG (H+L) (Invitrogen Life Technologies). Plates were observed using an inverted

fluorescent microscope to determine infected cells. Titers were determined using the Reed-Muench equation.[19]

IFN-γ ELISPOT

The IFN-γ ELISPOT was performed using a modification of previously described methods.[39] Briefly, Millipore Multi-Screen filter 96 well plates were pre-wet with 70% ethanol, washed with PBS, coated with purified mouse anti-swine IFN-γ (BD Biosciences), and incubated overnight at 4°C. Following incubation, plates were washed with RPMI media (Sigma-Aldrich) and blocked for 1 hour at room temperature with RPMI containing Fetal Bovine Serum and antibiotics (complete RPMI). After blocking, the media was decanted and each PBMC sample was plated in replicates at a concentration of $2x10^5$ PBMC/well. PBMC plus complete RPMI was used as the unstimulated negative control and PBMC plus PHA-P at 10μg/ml was used as the positive control. PBMC were plated in duplicates and then stimulated with whole virus H3N2 or pH1N1 supernatants at titers of $1x10^6$ $TCID_{50}$. PBMC collected on the day of challenge were also stimulated with NP RP at a titer of $1x10^6$ RP. The plates were incubated with the stimulating antigens for 18-22 hours at 37°C. After incubation, the plates were washed with 1x KPL wash solution. Biotinylated mouse anti-swine IFN-γ (BD Biosciences) was added to each well and incubated for 1 hour at 37°C. After washing, alkaline phosphate labeled streptavidin (Bio-Rad) was plated and incubated for 1 hour at 37°C. Plates were developed for 3 to 10 minutes using an alkaline phosphatase substrate kit (Bio-Rad). Positive spots were enumerated using a Zeiss ELISPOT reader system (Zellnet Consulting Inc.). The number of IFN-γ producing PBMC was determined by subtracting the number of spots from the wells with no antigen stimulation

from the wells stimulated with antigen. This number was then normalized to IFN-γ secreting cells/1x10^6 PBMC.

Statistical analysis

Analysis of variance (ANOVA) was used to analyze reciprocal HI titers, ELISA titers, ELISPOT counts, log_{10} transformed live virus titers, average daily gain, and gross and histopathological lung scores. Analyses were performed using the JMP software (SAS). Statistical significance was set at $p<0.05$.

Results

Homologous pH1N1 HA RP efficacy study

All pigs in the HA RP vaccinated group developed positive HI titers by 14 days post-boost vaccination and maintained similar titers until challenge, while all pigs in the placebo group remained seronegative throughout the entire study (Table 1). In addition to homologous HI titers, pandemic HA RP vaccination induced positive HI titers against other gamma-cluster H1N1 virus isolates (Table 1).

No live virus was detected in nasal swabs at any day post-challenge in the HA RP vaccinated group. In contrast, all five pigs in the placebo vaccinated group had detectable nasal swab virus titers at days 4 and 5 post-challenge (Table 2). Live virus was detected from all five BALF samples from the placebo vaccinated pigs, while only one BALF sample contained live virus in the HA RP vaccinated group (data not shown).

Macroscopic evaluation of lungs exhibited lesions consistent with SIV infection and consisted of variably sized, firm, and consolidated red foci located primarily in the cranioventral lung fields. The HA RP vaccinated pigs exhibited reduced lung lesion

scores when compared to placebo vaccinated pigs (Table 2). The lower macroscopic lung scores in the HA RP group correlated with the microscopic analysis and SIV IHC results. Lung samples taken from HA RP vaccinated pigs exhibited significantly less microscopic pneumonia than placebo vaccinated pigs (Table 2). All five lungs collected from the placebo pigs had detectable SIV antigen as demonstrated by IHC, while no SIV antigen was detected in any of the lungs taken from the HA RP vaccinated pigs. In addition to decreased pulmonary pathology, the HA RP vaccinated pigs demonstrated increased average daily gain post-challenge when compared to placebo vaccinated animals (Table 1).

NP RP efficacy study

The pigs in the NP RP vaccinated group developed robust antibody titers while all the pigs in the placebo vaccinated group remained seronegative throughout the study (Table 3). All of the pigs in the NP RP vaccinated group developed positive NP ELISA titers prior to booster vaccination. The NP antibody titers increased significantly following boost and remained at high levels until the day of challenge (Table 3).

Nucleoprotein RP vaccination also induced an antigen-specific CMI response as measured by the IFN-γ ELISPOT assay. By 21 days post-primary vaccination and again on the day of challenge, the pigs receiving the NP RP vaccine demonstrated a significantly elevated IFN-γ response to both H3N2 and pH1N1 influenza viruses when compared to the placebo vaccinated pigs. The group receiving the NP RP vaccine had a significantly higher number of IFN-γ secreting cells than the placebo vaccinated animals on the day of challenge when NP RP was used as the stimulating antigen.

No live virus was detected from the NP RP vaccinated pigs at 24 hours post-challenge, while two of the placebo vaccinated pigs were shedding live virus. By day 3 post-challenge all pigs in the placebo vaccinated group were shedding virus, and all pigs in the NP RP vaccinated group were shedding virus by day 4 post-challenge. There were no significant differences in the number of pigs between groups that were shedding virus at any day post-challenge. However, pigs that received the NP RP vaccine shed significantly less virus than placebo vaccinated pigs on days 3, 4, and 5 post-challenge. The BALF viral titers were also significantly lower in the NP RP vaccinated pigs than placebo vaccinated pigs.

The gross lesions observed at necropsy were consistent with typical SIV infection. These macroscopic lung scores were not significantly different between the two vaccination groups (data not shown).

Discussion

This is the first study to demonstrate the efficacy of an alphavirus RP vaccine against pH1N1 in pigs. Although RP vaccines expressing the HA protein have been evaluated previously,[2,7,14,21] this is the first study evaluating NP RP in pigs using a heterosubtypic vaccination challenge model. Studies have shown that homologous vaccination against the pH1N1 virus represents the most efficacious vaccine option, although there appears to be some cross-protection from non-homologous vaccines that contain influenza strains from the same phylogenetic cluster,[30] Those previous results correlate with the results reported here, and demonstrate that an HA RP vaccine is as effective as inactivated whole virus vaccination against pH1N1. The homologous HA RP

vaccine used in this study eliminated nasal shedding in all vaccinated animals, and also protected against pulmonary pathology. In addition, HA RP vaccinated animals demonstrated increased weight gain in the five days following virulent pH1N1 challenge, similar to a previous study evaluating alphavirus replicon-produced recombinant pH1N1 HA vaccines,[26] These results indicate that, in addition to eliminating nasal shedding and decreasing pulmonary pathology associated with pH1N1, HA RP vaccination can also increase the production performance of these economically important food animals.

Several studies have reported enhanced pneumonia following inactivated whole virus vaccination and subsequent heterologous challenge.[9,32] Although the reason for this phenomenon has not yet been elucidated, it is possible that the enhancement is the result of non-HA specific immune responses. Heinen et al. reported enhanced disease following M2e/NP DNA vaccination, and implicated M2e-specific antibodies in the exacerbation of clinical disease.[13] It is probable that enhancement of disease occurs to some extent in the field due to the widespread use of inactivated influenza vaccines in swine. Swine influenza virus vaccines expressing only the HA antigen may be able to circumvent this enhancement, but additional research with these vaccines is required to further elucidate the mechanism of enhancement.

In this study we report HI cross-reactivity to additional H1-gamma isolates following pH1N1 HA RP vaccination. Although this has been previously reported,[30,31] these results demonstrate that pH1N1 HA RP vaccination is able to offer some level of protection against H1-gamma SIV strains without the requirement for both gamma and pH1N1 strains to both be included in a single vaccination. In addition, there was no seroconversion to the vaccine antigen in any of the placebo vaccinated pigs in either

study, further confirming the lack of shed/spread, reversion to virulence, and overall safety of RP vaccines.

Current SIV vaccines often fail to protect against emerging strains and offer only limited protection against heterosubtypic challenge. This has resulted in the implementation of new procedures by the U.S Department of Agricultures' Center for Veterinary Biologics (USDA CVB) to expedite the licensure process required for the addition or substitution of new SIV strains to existing vaccines (Veterinary Services Memorandum No. 800.111), and has led to increased evaluation of broadly-protective or universal vaccines that offer heterosubtypic protection. In this study we vaccinated pigs with an RP vaccine expressing the NP gene derived from a cluster 4 H3N2 SIV isolate and challenged with the heterosubtypic pH1N1 influenza strain. Recombinant adenovirus vaccines expressing NP have been previously evaluated in both the presence and absence of maternal antibodies in young pigs.[34,35] Adenovirus-based NP vaccination alone decreased nasal shedding on days 1, 4, and 5 post-homosubtypic challenge when compared to non-vaccinated controls with no significant differences in gross lung lesions observed between the two groups.[35] A similar decrease in viral load with no differences in lung lesions was also observed in this study, but the challenge strain used here was heterosubtypic to the vaccine gene.

The mechanisms regarding NP-specific protection following heterosubtypic challenge have yet to be fully elucidated. Initially, it was shown that a specific CD8+ cytotoxic T lymphocyte (CTL) immune response was needed for heterosubtypic protection,[24,36] and eventually a role for cytokine-secreting CD4+ T cells was also demonstrated.[25] B cell-deficient mice primed with influenza virus develop enhanced

immunity following heterosubtypic challenge but this protection was no longer observed when the CTL population was depleted.[23] However, other studies have questioned the necessity of a CTL response for protection following NP vaccination and suggest a possible role for NP-specific antibodies.[1,6] A recent study demonstrated that antibody-deficient, T cell-competent mice were not protected against influenza challenge following NP vaccination, but these mice were protected after passive transfer of NP-specific antibody serum.[3] This protective role for anti-NP antibodies is in contrast with a previous study that demonstrated that transfer of anti-NP monoclonal antibody to *scid* mice (B and T cell-deficient) resulted in no protection from influenza challenge.[10] Together, these studies indicate that both functional B and T cells are required for efficient protection against heterosubtypic influenza. In the current study, we demonstrate both robust B and T cell responses following NP vaccination via NP-specific ELISA and IFN-γ ELISPOT, respectively. Again, placebo vaccinated pigs did not seroconvert to NP, demonstrating the safety of this vaccine platform. The IFN-γ CMI response was specific for both H3N2 and pH1N1 influenza viruses. Further, stimulation of PBMCs with NP RP resulted in an increase of IFN-γ secreting cells. This increase was not observed when PBMC collected from the PRRSV M RP group (placebo) were stimulated with NP RP, indicating that the increase in IFN-γ secreting cells was antigen-specific (NP) and not induced non-specifically by the alphavirus replicon platform. IFN-γ is the main cytokine released by CD8 effector T cells, and is also released by T_H1 CD4 T cells. Subsequent studies and flow analysis in our lab have shown that the majority of IFN-γ produced in pigs following RP vaccination is from the CD4+CD8+ T cell subset, and to a smaller percentage, CD4+CD8- and CD4-CD8+ subsets (data not shown). The

CD4+CD8+ subset is comprised of memory T cells, and has been shown to have a 5-10 fold higher frequency of IFN-γ producing cells than the other two lymphocyte populations in pigs.[38] Thus, this double positive T cell subset may play an important role in protecting pigs against heterosubtypic influenza infection.

In summary, the studies presented here demonstrate that the alphavirus replicon system can be used to produce RP vaccines that induce specific humoral and CMI responses against emerging viral diseases, and can also be utilized for the further development of influenza vaccines that offer heterosubtypic protection.

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Table 1. Study 1 hemagglutination inhibition (HI) geometric mean reciprocal titers and average daily gain (ADG) ± standard error of the mean (SEM)*.

Group	HI Titers				
	14 dpb[1]			26 dpb	
	pH1N1	H1N1 99[2]	Pfizer XP-012	pH1N1	ADG
Placebo	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	1.8 ± 0.2[a]
HA RP	121.3 ± 19.6[b]	60.6 ± 9.8[b]	34.8 ± 11.0[b]	91.9 ± 24.0[b]	2.5 ± 0.3[a]

*Values within a column not connected by the same superscript letter are significantly different ($p<0.05$).
[1]dpb: days post-booster vaccination
[2]Field SIV strain isolated in 1999 at Iowa State University Veterinary Diagnostic Lab (H1N1 γ-cluster)

Table 2. Study 1 mean $TCID_{50}$/ml log_{10} virus titers of nasal swabs (NS), gross and histopathological lung scores ± standard error of the mean (SEM)*.

Group	Log_{10} Virus Titers		Gross Lung Scores (%)	Histopathological Lung Scores (0-3)
	NS 4 dpc[1]	NS 5 dpc		
Placebo	3.1 ± 0.2[a]	3.1 ± 0.2[a]	15.6 ± 5.4[a]	1.8 ± 0.1[a]
HA RP	0.0 ± 0.0[b]	0.0 ± 0.0[b]	3.6 ± 3.1[a]	1.0 ± 0.0[b]

*Values within a column not connected by the same superscript letter are significantly different ($p<0.05$).
[1]dpc: days post-challenge

Table 3. Study 2 mean NP ELISA S/P values ± standard error of the mean (SEM). Values ≤0.67 are considered positive*.

Group	Multiscreen NP ELISA			
	Pre-Vac	0 dpb[1]	8 dpb	21 dpb
Placebo	0.82 ± 0.03[a]	1.01 ± 0.02[a]	0.93 ± 0.02[a]	0.90 ± 0.02[a]
NP RP	0.85 ± 0.02[a]	0.55 ± 0.03[b]	0.17 ± 0.02[b]	0.18 ± 0.01[b]

*Values within a column not connected by the same superscript letter are significantly different ($p<0.05$).
[1]dpb: days post-booster vaccination

Table 4. Study 2 mean IFN-γ secreting cells per $1x10^6$ PBMC ± standard error of the mean (SEM)*.

Group	Day of Boost		Day of Challenge		
	H3N2[1]	pH1N1	H3N2	pH1N1	NP RP
Placebo	23.3 ± 8.1[a]	19.0 ± 8.5[a]	45.5 ± 10.6[a]	39.3 ± 11.0[a]	26.0 ± 9.0[a]
NP RP	61.5 ± 9.6[b]	77.5 ± 14.2[b]	153.5 ± 51.5[b]	189.8 ± 62.7[b]	126.0 ± 43.4[b]

*Values within a column not connected by the same superscript letter are significantly different ($p<0.05$).
[1]Stimulating antigen used in ELISPOT assay

Table 5. Study 2 mean $TCID_{50}$/ml log_{10} virus titers of nasal swabs (NS) and bronchoalveolar lavage fluid (BALF) samples ± standard error of the mean (SEM)*.

Group	Log10 Virus Titers			
	NS 3 dpc[1]	NS 4 dpc	NS 5 dpc	BALF
Placebo	4.2 ± 0.3[a]	4.7 ± 0.1[a]	4.4 ± 0.2[a]	3.9 ± 0.5[a]
NP RP	2.8 ± 0.4[b]	4.1 ± 0.1[b]	3.3 ± 0.3[b]	1.6 ± 0.6[b]

*Values within a column not connected by the same superscript letter are significantly different ($p<0.05$).
[1]dpc: days post-challenge

CHAPTER 6. GENERAL CONCLUSIONS

The results presented in this dissertation support the conclusion that alphavirus-based replicon particle (RP) vaccines are immunogenic, efficacious, and safe for use in swine. Replicon particle vaccines have been evaluated extensively in both the human and veterinary fields; however, no replicon-based vaccine has been approved by a government regulatory agency to date, although several human candidate vaccines have been successfully evaluated in preclinical trials. This is the first report of a formal RP vaccine safety study in a veterinary species. When the RP vaccine was administered to pigs at high doses (200X efficacious dose) there was no detectable shed or spread of the vaccine from vaccinated animals to non-vaccinated cohorts. Additionally, no virulent virus was detected in any of the tissues samples, indicating a lack of reversion to virulence. These results provide the necessary evidence that RP are safe to use as veterinary vaccines and pose minimal risk to animals, humans, or the environment.

In addition to demonstrating safety, these studies show that RP vaccines are highly immunogenic in swine. Replicon particle vaccines expressing the hemagglutinin (HA) gene from both H3N2 and pandemic H1N1 (pH1N1) influenza strains resulted in specific humoral and cell-mediated immune responses. Following homologous influenza challenge, pigs were completely protected against nasal shedding and exhibited reduced pulmonary pathology. In addition, replicon-based vaccines increased the average daily gain of vaccinated pigs following influenza challenge, resulting in an increase in the economic value of vaccinated animals. This research also demonstrates the speed with which RP vaccines can be produced in response to emerging diseases. When the pH1N1

influenza virus was first reported in the human population, we were able to produce a replicon-based vaccine in two months. Because the viral isolate is not required for vaccine production but only a protective gene sequence, production can occur more rapidly than for traditional vaccines. The quick response demonstrated in this study could someday be implemented to produce vaccines against foreign or emerging diseases (i.e. foot-and-mouth disease virus).

Influenza viruses are constantly evolving, and currently many different subtypes and clusters co-circulate among the U.S. swine population.[8-10] There is little antigenic cross-reactivity between isolates from these different subtypes and clusters.[1-3,8] Thus, broadly-protective vaccines must either be multivalent or contain antigens that are immunogenic and conserved among all influenza strains. As discussed in Chapter 2, multivalent RP vaccines have been shown to be immunogenic and efficacious against multiple pathogens.[6,7] Thus, the RP technology can be utilized to produce a multivalent vaccine that expresses protective antigens from multiple relevant SIV strains. The other approach for a broadly-protective SIV vaccine is to focus the immune response against a conserved antigen. This approach was evaluated in Chapter 5 by administering a NP RP vaccine followed by a heterologous challenge with pH1N1. The NP RP vaccine was highly immunogenic and induced both humoral and cell-mediated responses, and following heterologous pH1N1 challenge, NP RP vaccinated animals demonstrated reduced nasal shedding. These results indicate that, although not as efficacious as homologous HA RP vaccination, NP RP vaccines are able to decrease viral load and may therefore be an important component of a universal influenza vaccine. Such vaccines could play an important role in reducing future outbreaks of influenza in pigs from non-

vaccine matched strains. Vaccines capable of protecting against new strains are also important for reducing the zoonotic risk of swine influenza viruses.

These studies demonstrate that alphavirus-based RP are good candidates for swine vaccines. However, swine RP vaccine research is still in its infancy with many questions or hypotheses that remain to be tested. First, can an efficacious multivalent SIV RP vaccine be developed that offers protection against multiple subtypes and clusters? Based on results from previous studies, multivalent RP vaccines have been shown to be as effective as the monovalent components of such vaccines.[4-6] Therefore, there is a reasonable expectation that such a multivalent SIV RP will provide comparable levels of protection against all strains as the monovalent H3N2 HA RP vaccine. The alphavirus vector can also be easily manipulated to allow several genes to be inserted on a single replicon RNA, thus simplifying vaccine production and reducing production costs.

The results presented in Chapter 5 indicate that NP RP vaccination reduces the level of virus shedding. How can these promising results be further expanded to develop a more efficacious broadly-reactive SIV vaccine? Other studies have demonstrated broadly-protective immune responses utilizing a novel prime-boost strategy of an HA adenovirus-vectored vaccine followed by an inactivated influenza vaccine.[11] Additionally, an adenovirus vector vaccine expressing HA and NP, when used in a prime-boost regimen with an inactivated vaccine, was able to protect young pigs in the presence of maternal antibody.[12] Thus, it is conceivable that by developing an RP vaccine expressing both NP and HA that even broader protection could be achieved, especially when used in conjunction with novel prime-boost strategies. An SIV RP vaccine that is able to induce broadly-protective immunity and perhaps even provide

protection in the presence of maternal antibodies should be further evaluated. Such a vaccine would be very beneficial to the swine industry and would represent a proactive approach to preventing further influenza outbreaks.

Finally, since the studies presented here demonstrate that RP vaccination of swine is safe and efficacious and due to the relative resistance to antivector immunity, RP vaccines against other swine pathogens should be considered. As described in chapter 4, RP vaccines are not shed or spread and do not revert to virulence. This is a tremendous benefit when compared to traditional modified live vaccines. Also, other studies have already demonstrated that animals can be immunized multiple times with the same RP vaccine or with a different RP vaccine with no effect on the host immune response to booster immunizations.[7] Protective genes from multiple pathogens can also be included on one replicon RNA, thereby decreasing the number of injections required. Therefore, multivalent RP vaccines should be further developed for SIV and other important swine pathogens.

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