DEVELOPMENT OF PARAPOXVIRUS ORF VIRUS AS A VACCINE DELIVERY PLATFORM FOR USE IN SWINE

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DEVELOPMENT OF PARAPOXVIRUS ORF VIRUS AS A VACCINE DELIVERY PLATFORM FOR USE IN SWINE

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Orf virus (ORFV) is the member of the parapoxvirus genus within the *Poxviridae* family. Efforts are being made to develop orf virus as a vaccine delivery platform for use in swine. The studies presented in this dissertation are aimed at developing orf virus-based vectored vaccines against different viral pathogens of swine and to improve the immunogenicity of the orf virus vector. Our previous studies have shown that ORFV recombinant expressing spike protein of porcine epidemic diarrhea virus (OV-PEDV-S) can provide protection to pigs from the virulent PEDV challenge. To further investigate if OV-PEDV-S recombinant can induce immunity in sows that is transferred to newborn piglets with the colostrum/milk and can protect the piglets against challenge infection with PEDV, an animal experiment was conducted where pregnant sows were immunized with OV-PEDV-S and piglets born from the immunized sows were challenged with PEDV. OV-PEDV-S was able to induce maternal immunity in piglets and there was significantly less mortality (<5% mortality) in the piglets born from the immunized sows when compared to the piglets born from non-immunized sows (50% mortality). Additionally, ORFV recombinants expressing hemagglutinin protein of influenza virus (OV-HA) and a recombinant expressing hemagglutinin and

nucleoprotein of influenza virus (OV-HA-NP) were developed and their immunogenicity and protective efficacy was assessed in pigs. These recombinants induced robust humoral and cell-mediated immune response against swine influenza in pigs and when challenged with virulent H1N1 swine influenza virus, pigs immunized with OV-HA and OV-HA-NP were protected from the clinical disease. Another study was conducted to identify novel orf virus promoters using a transcriptomic approach. We demonstrate that an endogenous orf virus promoter, p116, leads to robust expression of heterologous genes *in vitro*. Overall, data presented in this paper provide evidence that orf virus could be an excellent vaccine delivery platform for use in swine. And the novel promoters identified here could improve the immunogenicity of ORFV recombinants by increasing level of heterologous gene expression.

BIOGRAPHICAL SKETCH

Lok Raj Joshi was born in Kailali, Nepal. He completed his schoolwork from Dhangadhi, Kailali. After completing his schoolwork from Galaxy Secondary School, Lok joined National School of Sciences in Kathmandu, Nepal to complete his high schoolwork with a science major. After completing his high school, Lok attended Institute of Agriculture and Animal Science (IAAS, Tribhuvan University) in 2008 to obtain Bachelor of Veterinary Science and Animal Husbandry (B.V. Sc & A.H, equivalent to DVM). Lok was awarded meritorious student scholarship at IAAS, which covers the cost of attending veterinary school. Lok completed his veterinary degree in 2013. Lok was involved in various research activities while he was attending vet school in Nepal. Ho moved to the United States for further education in January of 2015. He joined the lab of Dr. Diego Diel at South Dakota State University, Brookings, South Dakota in 2015 and obtained master's degree in Biological Science in 2017. He worked on the pathogenesis and molecular evolution of Senecavirus A during his master's degree. He continued his PhD degree in Diel Lab and in 2019 he joined Biological and Biomedical Science (BBS) PhD program at Cornell University. His PhD work involves developing orf virus-based vaccine delivery platform for use in swine.

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LIST OF ABBREVIATIONS

BFA	Brefeldin A
CBP	Chemokine binding protein
CFSE	Carboxyfluorescin succinimidyl ester
CNPV	Canarypox virus
CTL	Cytotoxic T-lymphocytes
CWPV	Cowpox virus
DPC	Days post-challenge
DPI	Days post-immunization
ELISA	Enzyme linked immunosorbent assay
FFN	Fluorescent focus neutralization assay
FWPV	Fowlpox virus
GFP	Green fluorescent protein
HA	Hemagglutinin protein of influenza virus
HI	Hemagglutination inhibition
IACUC	Institutional Animal Care and Use Committee
IAV-S	Influenza A virus of swine
ICS	Intracellular cytokine staining
IFA	Immunofluorescence assay
IFN-γ	Interferon gamma
IL-4	Interleukin 4
IM	Intramuscular
IMP	Immunomodulatory proteins
MDCK	Madin-Darby canine kidney cells
MEM	Minimum essential media

MOI	Multiplicity of infection
NA	Neutralizing antibody
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NP	Nucleoprotein of influenza virus
OFTu	Ovine fetal turbinate cells
ORF	Open reading frame
ORFV	Orf virus
OV-HA	Orf virus expressing hemagglutinin of swine influenza virus
OV-HA-NP	Orf virus expressing HA and nucleoprotein of swime influenza
OV-IA82	Orf virus IA82 strain
OV-PEDV-S	Orf virus expressing spike protein of porcine epidemic diarrhea virus
PBMC	Peripheral blood mononuclear cells
PEDV	Porcine epidemic diarrhea virus
RabV G	Rabies virus glycoprotein
RT-PCR	Reverse transcriptase polymerase chain reaction
STU	Swine turbinate cells
TCID50	Tissue culture infectious dose 50
TMB	3,3′,5,5′-tetramethylbenzidine
VACV	Vaccinia virus
VGEF	Vascular endothelial growth factor
VN	Virus neutralization

CHAPTER 1

A review on poxvirus vectors

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Abstract

The utility of poxviruses as an expression vector was first described in early 1980s. Since then, poxviruses have been widely used as a vaccine delivery platform in human and veterinary medicine. The main features of poxviruses that make them an excellent vector candidate are their large genome size with the presence of multiple immunomodulatory genes, tolerance for large heterologous inserts and ability to induce cellular and humoral immunity. Initial attempts were focused on engineering vaccinia virus to express heterologous gene. Later, the potential of other poxviruses like avipoxvirus, parapoxvirus and swinepoxvirus to be used as vectors were also explored with promising results. To address the safety concerns related to wild type poxviruses, several highly attenuated, replication-defective strains have been developed mostly by serial passages in cell culture. Most of the poxvirus recombinants developed till now have focused on insertional inactivation of thymidine kinase (TK) gene where heterologous gene is inserted at TK locus. In recent years, other immunomodulatory genes have also been used to generate safer and multivalent vectored vaccines. Homologous recombination is the method of choice for the construction of poxvirus recombinants. Lately, there has been significant improvement in recombinant selection methods like using fluorescent protein, using CRISPR tools. Poxvirus vectors have been shown to be very effective in heterologous prime-boost immunization regimes, where poxvirus vectors are used in combination with other killed or DNA vaccines. To date multiple poxviruses have been developed and some of them have been licensed for use in variety of pathogens like rabies virus, avian influenza virus, canine distemper virus, West Nile virus. Here, the use of poxviruses as a vector for use in veterinary medicine has been described in detail.

Introduction

Poxviruses are large complex viruses that belong to the family *Poxviridae*. The *Poxviridae* is divided in two sub-families the *Chrodopoxvirinae* and *Entomopoxvirinae*. The sub-family *Chordopoxvirinae* comprise viruses that infect vertebrate animal species whereas the *Entomopoxvirinae* contains viruses that mainly infect invertebrate insects. Currently, there are 11 genera classified under the *Chordopoxvirinae* subfamily and 3 genera under the *Entomopoxvirinae* [1]. Poxviruses are classified in these genera on the basis of virus morphology, phylogeny, serological cross-reactivity and host-range [2]. The poxvirus species name usually refers to the host from which virus was first isolated. While some poxviruses have restricted host range (i.e. variola virus known to infect only humans), there are many other, including cowpox virus (CPXV), buffalopox virus (BPXV), monkeypox virus (MPXV) which have broad animal host range infecting multiple mammalian species including humans. Poxviral infections occur through different routes, including the skin (Orf virus), respiratory tract (variola virus), or oral route (ectromelia virus) [3]. Poxvirus infections are characterized by formation of skin lesions, usually evolving through the stages of papules, pustules, vesicles, nodules and scabs [4]. Poxviruses are among the largest known viruses. Most poxviruses contain brick-shaped virions

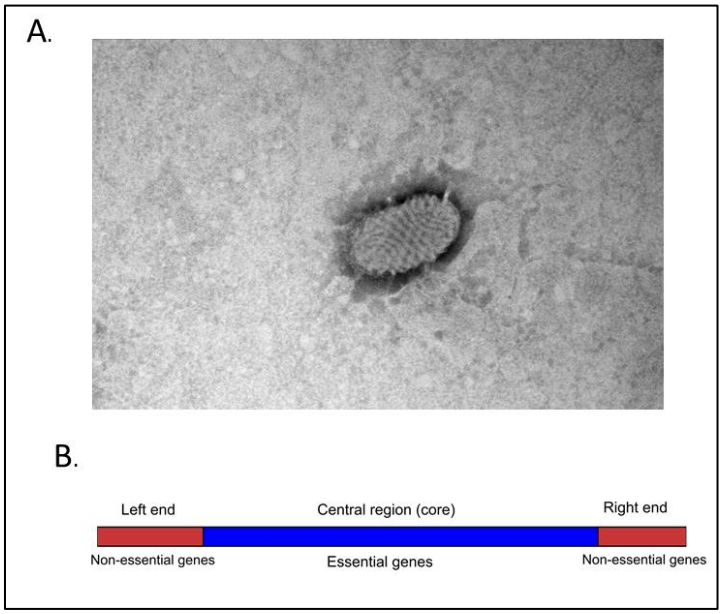

Figure 1.1. Structure and genome of poxvirus. (A) Negative stain preparation of Orf virus (parapoxvirus). (B) Schematic representation of poxvirus genome. The essential genes are present in the central part of the genome. The non-essential genes which play role in immunomodulation are present in either end of the genome.

with a particle size ranging from 220–450 nm long × 140–260 nm wide × 140–260 nm thick (Figure 1.1A). Parapoxviruses are oval-shaped with a particle size of 260 nm × 160 nm [4]. Virions are enveloped with the presence of surface tubules or surface filaments **(**Figure 1.1B). Internally, the virions contain two lateral bodies and a dumb-bell shaped nucleoprotein core. The nucleoprotein core contains enzymes essential for virus replication and the nucleocapsid protein bound to the viral genome. The viral genome is linear double-stranded DNA with the size ranging from 130 kbp to 375 kbp and encode between ~130 - 350 open reading frames (ORFs). The two strands of DNA are cross-linked at the termini due to presence of A+T-rich inverted terminal repeats (ITR) at the two ends of the genome. The central region of the genome is highly conserved across different poxviruses and encodes genes essential for viral transcription, replication and virion assembly. Non-conserved genes that are involved in virus host-range, immunomodulation and pathogenesis are present at either end of the genome, flanking the conserved central genome core (Figure 1.1B).

A unique feature of poxviruses is their replication site, which takes place in the cytoplasm of infected cells, making poxviruses an exception among DNA viruses. The replication mechanisms of vaccinia virus (VACV) have been widely studied and most of our understanding of poxvirus replication comes from VACV. Notably, transcription and expression of poxviral genes is temporally regulated, and the genes are classified as early, intermediate or late genes based on the time of expression in relation to virus genome replication. In general, early genes are transcribed before replication whereas intermediate and late genes are transcribed after the virus genome has been replicated. For VACV, for example, early, intermediate and late genes are expressed in 20, 100 and 140 min after infection, respectively [5]. The poxvirus virion contains essential enzymes to initiate viral transcription upon infection. Therefore, early genes are

transcribed within the virion core soon after the virus enters the cell and mRNAs are extruded into the cytoplasm for translation. These early genes encode for transcription factors required for expression of intermediate genes. Some of the early proteins also play important roles in host immune evasion and modulation. Once early genes are expressed, uncoating of the virion core takes place and DNA is released into the cytoplasm followed by viral DNA replication which occurs in discrete replication sites within the cytoplasm designated viral factories. The intermediate genes are expressed after DNA replication and encode for transcription factors required for the expression of late genes. The late genes encode proteins essential for virion assembly and early gene transcription factors which will be packaged within the virion core. After virus assembly, enveloped virions are released by budding whereas non-enveloped virions are released by cell lysis. These basic biological properties of poxviruses are critical features that need to be considered when designing poxvirus vectors.

Construction of poxvirus vectors

Poxviruses hold a unique place in the history of immunization. In 1976, Edward Jenner demonstrated that smallpox could be prevented by using CWPV as a vaccine [6]. Later, VACV was widely used to immunize people against smallpox, which culminated with the eradication of the disease in 1980. To date, smallpox remains the only human disease that has been eradicated. Although, smallpox was eradicated, and vaccination was discontinued, the biological and immunomodulatory properties of VACV, the virus used as vaccine against smallpox, generated significant interest in poxviruses among scientists worldwide. Soon after the eradication of smallpox, a few studies describing genetically engineered VACV and the use of vaccinia as an eukaryotic expression system were published [7, 8]. In addition, recombinant VACV expressing single or multiple heterologous viral antigens were developed establishing the foundation for the

use of poxvirus as vaccine delivery vectors [9–11].

Some of the features that made VACV a well-received and widely used vector are: i. the large genome size (139 kb), with the presence of many non-essential genes, which could be manipulated without severely impacting virus replication; ii. the ability of VACV to tolerate insertion of up to 25,000 bp of foreign DNA [12]; iii. the fact that the virus is a potent inducer of both humoral and cell mediated immunity [13]; iv. the ease of administration and its efficacy through different immunization routes [13]; and v. the stability of the virus at room temperature when lyophilized, which obviates the need for cold chain [14]. Given that poxviruses share many common properties, the features described for VACV above also apply to other viruses in the family. The immunomodulatory properties of poxviruses and the efficacy of VACV as a vector platform, led several groups to explore other poxvirus vector alternatives. Several studies showed the potential of other members of the family *Poxviridae,* including avipox viruses (fowlpox [FWPV] and canarypox virus [CNPV]), swinepox virus (SWPV), and Orf virus (ORFV), as vectors for human and veterinary applications. These vectors are described below with the primary focus on their use to deliver veterinary vaccines.

Strategies used in developing recombinant poxvirus vectors.

Earlier studies in the 1960s showed that genetic recombination can occur between two different strains of related poxviruses when both viruses infect a single cell [15, 16]. This process known as homologous recombination involves the exchange of nucleotide sequences between two similar or identical DNA molecules [17]. Homologous recombination is now widely used for the generation of recombinant poxviruses [18–21]. This method requires the construction of a transfer plasmid (recombination plasmid) containing the foreign gene insert (heterologous gene) and the left- and right-homology DNA sequences flanking the insertion site from the parent poxvirus

genome (Figure 1.2). Homologous recombination and recombinant poxvirus generation are achieved by infecting permissive cells with the parent poxvirus and subsequently transfecting these cells with the recombination plasmid (infection/transfection). Within cells that were infected and transfected, homologous recombination between the parental virus and the recombination plasmid takes place, resulting in a new chimeric recombinant poxvirus. The recombinant poxvirus is purified by multiple rounds of limiting dilution and/or plaque assay (Figure 1.2).

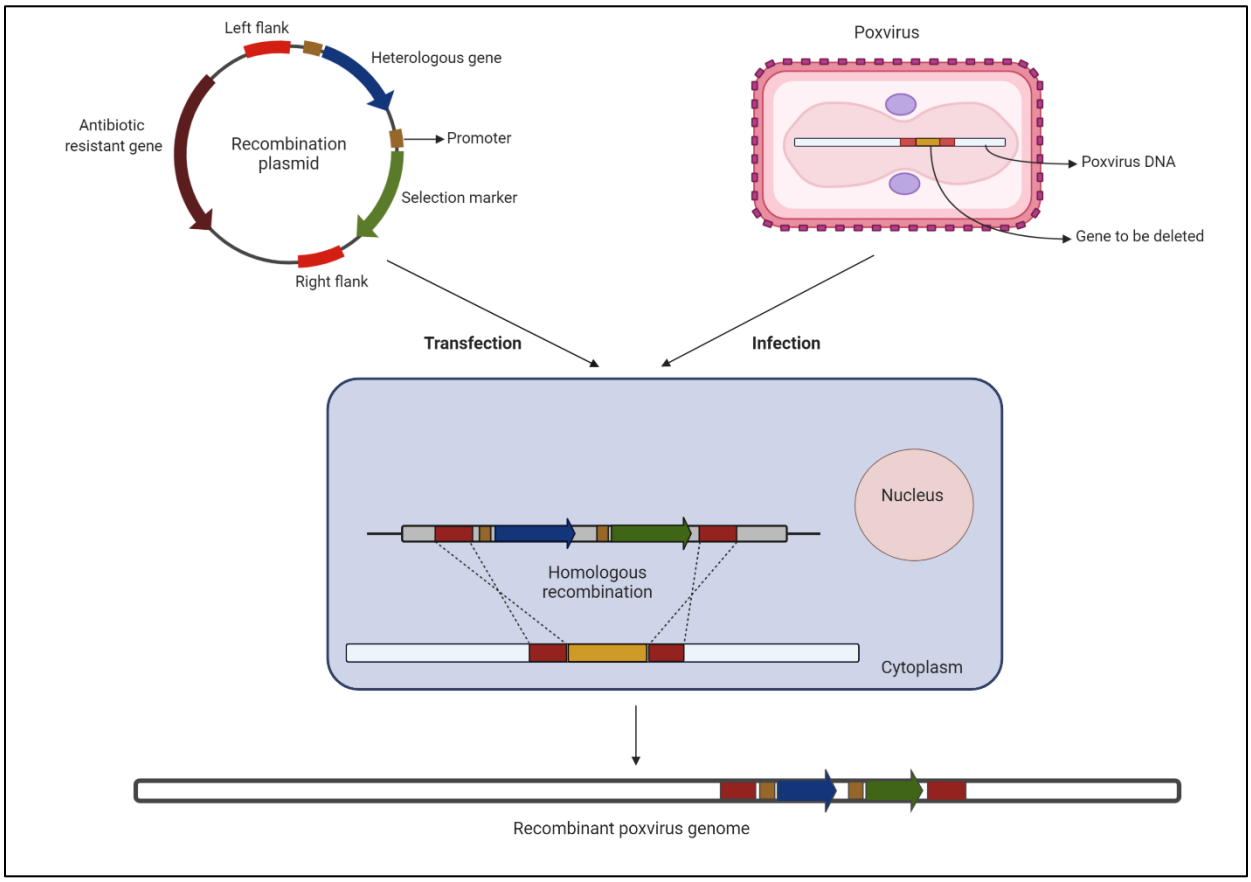

Figure 1.2 Schematic representation of homologous recombination. Cells are infected with parental poxvirus and transfected with recombination plasmid. Homologous recombination between the plasmid and parental poxvirus genome occurs within the cytoplasm resulting in a chimeric recombinant poxvirus.

There are several factors that need to be considered to achieve homologous recombination, including the homology length and the DNA structure [22]. Higher recombination frequencies

were obtained, for example, when homologous flanks with at least 100-350 bp and linear plasmid DNA were used in infection/transfection experiments with VACV [22]. In addition to the insertion site and homology length, the following factors need to be considered to design and generate poxvirus-based vectors:

1) **Promoters.** Given the temporal regulation of poxvirus gene transcription (early, intermediate and late), selection of the promoter that will drive expression of the heterologous gene is a critical aspect of the design of poxvirus vectors. In general, promoters with both early and late activity are ideal for expression of foreign genes because they drive expression of the heterologous genes throughout the vector infection cycle, promoting sustained expression of the antigen and consequent stimulation of the immune system. Early promoters would also be preferable when the poxvirus vector is replication defective or when the vector is to be used in a non-permissive animal species, both of which preclude expression driven by late promoter, which takes place virus after replication. The most commonly used promoters to drive expression of heterologous genes by poxviruses include the native VACV early/late promoters ($P_{7.5}$ or $VV_{7.5}$), the modified early promoter (mH5) or yet synthetic promoters such as PrS, for which expression has been optimized by mutagenesis [23].
2) **Termination signal.** The presence of poxvirus early termination signal TTTTTTNT within the sequence of heterologous genes could potentially lead to premature transcription termination, and consequently low expression levels or expression of a truncated protein [24]. Therefore, termination signals should be removed through site directed mutagenesis or synthetic biology from the heterologous gene sequence before inserting the gene into the vector [25].

3) **Codon optimization.** Codon optimization of the heterologous gene may help to achieve higher expression levels especially when the recombinant is to be used in non-target animal species, in which replication and late gene expression are impaired. Codon optimization helps in the stability of the recombinant vector by removing non-desirable sequences [23]. Additionally, it may also be used when multivalent heterosubtypic viral vectors containing two or more viral genes from closely related virus strains are designed. Codon optimization and changes in the nt sequence of one of the genes increases the stability of the vector by preventing or reducing the risk of intramolecular homologous recombination.

4) **Selection method**. Selection of recombinant poxviruses is one of the most time-consuming steps in generating recombinant poxvirus-based vectors. Conventionally, selection of recombinant poxviruses has been based on expression of the β-galactosidase reporter gene. The 5-bromo-4-chloro-indolyl-D-galactopyranosidase (X-gal) substrate is incorporated into the agarose overlay during plaque assay and recombinants expressing β-galactosidase form blue plaques, which can be selectively picked and purified [18, 26]. Additionally, drug resistance genes like neomycin resistance gene can be used as a selectable marker for selection and isolation of poxvirus recombinants [27]. More recently, fluorescent proteins like the green-fluorescent protein (GFP) have also been used successfully in recombinant poxvirus selection. The gene expressing GFP or other fluorescent-protein is inserted along with the gene of interest. The recombinant poxvirus expressing fluorescent protein can be selected by using plaque assay [20]. The presence of marker genes is not always recommended, as tandem expression of multiple genes can result in lower protein expression levels due to promoter interference. Therefore, strategies to develop markerless recombinant poxviruses have been recently developed. The most straightforward approaches involve selection of recombinant

viruses by real-time PCR or immunofluorescence assays targeting the heterologous genes [28]. Whereas, more sophisticated approaches using excisable marker systems based on Cre/loxP recombination, which facilitate selection and subsequent removal of marker gene, were also developed and provide an efficient means to create markerless recombinants [29]. Recently, a marker-free system for construction of vaccinia virus vectors using CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 has also been reported [30].

Application of poxvirus vectors

Several poxvirus platforms have been developed and used as vaccine delivery vectors in veterinary species. Below we present a brief discussion of the main poxvirus vectors and their applications and/or uses in animals.

Orthopoxvirus based veterinary vaccines

Vaccinia virus (VACV), the type species of the *Othopoxvirus* genus, has been widely used as a vector for vaccine delivery. Initially, parental moderately virulent VACV strains like western reserve (WR), Copenhagen and Lister were used to develop recombinant vaccines. However, safety concerns with the use of these strains were raised especially in immunocompromised hosts which usually experienced moderate-to-severe adverse vaccine reactions [31]. These limitations led to the development of highly attenuated VACV strains. For example, the VACV strain LC16m8 was developed by sequential passage of the Lister strain in primary rabbit kidney (PRK) cells at 30° C [32]. The modified vaccinia Ankara (MVA) has been developed by passage of VACV strain Ankara in chicken embryo fibroblasts (CEF) for 516 times [33]. The resulting virus lost ~15% of its genome during cell passaging [34] and it is replication deficient in most mammalian cells [35]. Another highly attenuated vaccinia virus strain NYVAC was derived from plaque-cloned isolate of the Copenhagen vaccine strain which contains select deletion of 15 non-

essential genes [36]. The NYVAC strain is less pathogenic and has greatly reduced ability to replicate in variety of mammalian cells (human, mice and equine cells) but it retained the ability to induce immune response [37]. Although, most of these highly attenuated vaccinia virus strains are known for their safety profile, their immunogenicity is often compromised due to high level of attenuation [38]. For example, higher doses or multiple doses of MVA-based vectored vaccines are required to achieve immune responses similar to wild type VACV strains [39]. Nevertheless, both parental (e.g. Copenhagen, WR) and highly attenuated strains (e.g. LC16m8, MVA, NYCAV) have been used to develop recombinant vectored vaccines for veterinary use.

The first recombinant poxvirus licensed to be used as vaccine is a VACV-based vectored vaccine for rabies. This recombinant was constructed by inserting the rabies virus (RabV) glycoprotein (G) gene in the thymidine kinase (TK) locus of the Copenhagen strain of vaccinia virus [40, 41]. It has been used to control rabies in red foxes in several European countries, in coyotes and raccoons in the USA and in raccoons in Canada [42, 43]. The vaccine is used as an oral bait which is dispersed in the wild habitat of the target species by hand or airplanes. This vaccine is safe and effective in foxes, raccoons and coyotes [44–46] . It has been shown to be effective in vampire bats which are important reservoir for rabies virus [47]. However, it is less effective in skunks and in dogs when administered orally [42, 48, 49] . Also, as it is live attenuated vaccine, safety concerns regarding exposure of live-virus based vaccine to non-target species have been raised. To develop safer alternatives to this vaccine, recombinant MVA, a highly-attenuated VACV strain, expressing the RabV G was developed [39]. This recombinant vector was immunogenic in mice, dogs and raccoons upon parenteral immunization. However, it was less immunogenic than the VACV-Copenhagen-based recombinant vector and required a higher dose to induce immune response equivalent to the Copenhagen-based recombinant. Furthermore, the

MVA recombinant failed to induce humoral immune response when immunized orally making it unsuitable to use in wild animal populations [39]. These observations highlight the fact that there is a fine balance between protective efficacy and attenuation of poxvirus vectors.

Recombinant VACV vectors expressing the haemagglutinin (H) and fusion (F) proteins of Rinderpest virus have been developed. Two vaccinia recombinants were generated by inserting the H or F gene into the TK locus of VACV Weyth strain. Immunization of cattle with either recombinant or with the mixture of the two recombinants provided 100% protection even when the immunized animals were challenged with 1000 times the lethal dose of Rinderpest virus [50]. There was no transmission of the VACV-vector from vaccinated animals to contact animals. Moreover, cattle vaccinated with the mixture of recombinants vectors presented solid immunity as indicated by absence of amnestic response after challenge infection with Rinderpest virus [50]. The immunized animals, however, developed pock lesions at the site of immunization indicating that the vector was not completely attenuated. Additionally, the use and production of the mixed vector formulation, containing equivalent doses of two different recombinants, was cumbersome [51].

To address these drawbacks, a recombinant of VACV strain Weyth expressing both H and F genes (vRVFH) was developed. The H gene was inserted into TK locus and F gene was inserted into HA locus in the VACV genome. The insertional inactivation of TK and HA genes led to further attenuation of the vector. Consequently, no pock lesions were observed after intradermal immunization in cattle. The protective efficacy of the vaccine was not affected and sterilizing immunity was observed in cattle against Rinderpest virus challenge [51]. Later, another VACV-based recombinant expressing H and F genes (v2RVFH) was constructed using TK locus of the VACV Copenhagen strain. A strong synthetic VV promoter was used instead of the natural $P_{7.5}$

VV promoter which was used in previous constructs. This resulted in three-fold increase in the expression level of H and F genes as compared to vRVFH. Intramuscular vaccination of v2RVFH with a dose of 10^8 PFU provided sterilizing immunity in cattle for at least 16 months [52].

Interestingly, VACV strain Weyth expressing H and F genes of rinderpest virus (vRVFH) provides protection to goats against peste-de-petitis virus (PPRV) challenge [53]. Despite the inability of vRVFH to induce anti-PPRV neutralizing antibodies, complete protection was observed in goats against PPR [53]. Cell-mediated immunity or non-neutralizing antibodies might be responsible for the protection elicited by this recombinant vector in goats. Similarly, cross-protection has also been demonstrated for canine distemper virus (CDV) vectored by VACV vectors. Vaccinia virus recombinants expressing either the measles virus fusion (F) or hemagglutinin (H) glycoprotein have been shown to protect dogs against CDV [54]. The recombinants were generated by inserting measles virus F or H gene in the TK locus of VACV Copenhagen strain and using the H6 synthetic promoter. These recombinants fail to induce CDV-neutralizing antibodies in dogs. However, inoculation of dogs with the recombinant VACV virus expressing H gene or co-immunization with the recombinant VACV expressing H and the recombinant expressing F protein was shown to protect dogs from lethal CDV challenge [54].

Vaccinia virus recombinant expressing glycoprotein (G) gene of vesicular stomatitis virus (VSV) induces protective neutralizing antibody responses in cattle. Neutralizing antibody levels increased by several fold after boosting. This recombinant provides partial protection to VSV challenge in cattle and protection is correlated with neutralizing antibody levels [55]. The utility of VACV-based vectors in animals has also been demonstrated in chickens. A VACV expressing the Newcastle disease virus (NDV) F glycoprotein has been shown to protect chickens against live virulent NDV challenge [56]

Recombinant VACV-vectors have also been used to develop vaccine candidates against protozoan parasites. A VACV expressing the LACK protein of Leishmania using the Western Reserve (rVV-LACK) or MVA (MVA-LACK) strains have been developed [57]. Prime-boost immunization of dogs with a plasmid DNA expressing the LACK protein (DNA vaccination) followed by booster with rVV-LACK or MVA-LACK incudes both humoral and cellular immunity in dogs. Priming with DNA and boosting with rVV-LACK provided 50% protection in dogs whereas boosting with MVA-LACK provided 75% protection in dogs. This study showed that boosting with non-replicative MVA vector elicited higher immune response than replication competent western reserve (WR) vector [57]. Later, another MVA construct expressing TRYP protein of Leishmania was developed [58, 59]. Dogs receiving a DNA-TRYP/MVA-TRYP prime boost vaccination strategy produced higher levels of TRYP specific type-1 cytokine IFN-gamma and TRYP specific IgG antibody in comparison to MVA-LACK construct [58]. These studies provided evidence for applicability of poxvirus vectors in prime boost vaccination regimens. In fact, VACV can be used for either priming or boosting. This has been demonstrated for a VACV recombinant expressing Gn and Gc glycoproteins of Rift valley fever virus (RVFV) [38]. The recombinant VACV-vector was generated using the Copenhagen strain and two virulence genes (B8R and TK) of the virus were inactivated. The RVFV Gn and Gc proteins were inserted in TK locus. A single vaccination with this recombinant provided 50% protection in mice, whereas animals immunized twice with this vaccine showed 90% survival rate after challenge. This recombinant was also tested in baboons (non-human primate model). All animals immunized with this recombinant mounted a strong anamnestic response to booster immunization [38].

Avipoxvirus as vectors

Avipoxviruses naturally infect chickens, turkeys and many other species of pet and wild

birds. Currently there are 10 species of avipoxviruses recognized by ICTV [60]. Given that avipoxviruses have been isolated from a wide range of hosts including crows, peacock, and ostrich among others; there are many avipoxviruses which have been tentatively proposed as new species but remain officially unclassified [61]. Avipoxviruses share many characteristics with other poxviruses. Our understanding of molecular and biological characteristics of avipoxviruses comes mainly from fowlpox virus (FWPV) and canarypox virus (CNPV), which infect domestic poultry and canaries, respectively [62].

As described in the previous section, VACV has been widely used as a vaccine vector for human and veterinary applications. However, VACV based recombinant vaccines developed for animals pose a risk of infection to humans because of the virus broad host range. Thus, it was desirable to construct host-restricted vectors, for instance, using recombinant avipoxviruses for use in mammalian species as these viruses only cause productive infection and disease in avian species [62]. The use of host-restricted or replication incompetent poxvirus vectors would avoid the risk of genetic recombination and disease transmission between vaccinated animal species or humans. Avipoxviruses vectors were initially proposed as vectors for vaccine delivery in poultry [63]. Later, the findings that recombinant FWPV initiate an abortive infection in non-avian tissue culture cells and express foreign antigens capable of inducing immune response in mammals sparked interest in using avipoxviruses as vectors for humans and other animal species [64]. Additionally, pre-existing immunity to orthopoxviruses does not affect immunogenicity of FWPV and canarypox virus (CNPV), which means they could be used as vectors in humans exposed to vaccinia virus or vaccinated against smallpox [61]. As a result, a large number of avipoxvirus recombinants based on FWPV and CNPV, have been developed for use in humans and animals.

Fowlpox virus-based vectors

Several fowlpox virus recombinant constructs targeting avian influenza (AI) have been developed. A fowlpox virus recombinant expressing the influenza virus HA protein at the TK locus was able to induce haemagglutinin inhibiting (HI) antibodies in chickens. These antibodies were detected as early as 9 days post-immunization and a boost effect was seen when chickens were re-immunized [63]. Interestingly, protection against AI has been observed in birds in presence of very low levels- or even the absence of HI or neutralizing antibodies [65]. Cell mediated immunity induced by immunization with the FWPV-vector has been suggested as the effector mechanism of protection against AI in birds without significant levels of HI or neutralizing antibodies [65]. To enhance cell mediated immunity induced by FWPV vectors, attempts were made to insert nucleoprotein gene (NP) of influenza virus along with HA gene. The co-expression of HA and NP by the FWPV vector, however, did not improve the efficacy of vaccine [66]. The FWPV-vectored avian influenza vaccine known as TROVAC-H5 has been licensed for emergency use in US and has full registration in Mexico, Guatemala, El Salvador and Vietnam [67]. The vaccine consists of FWPV recombinant expressing the H5 gene from highly pathogenic AI isolate A/turkey/Ireland/1378/83 H5N8. This vaccine provides 90%-100% protection against mortality against highly pathogenic Mexican avian influenza H5N8-type isolates. After a single immunization on day-one of life, it confers protection for at least 20 weeks [68]. Good levels of protection have also been observed against some of the recent H5N1 Asian AI isolates A/chicken/South Korea/03 and A/chicken/Vietnam/04 [67].

Another recombinant FWPV vector co-expressing HA (H5 subtype) and neuraminidase (N1 subtype) can provide complete protection to chickens against AI H5N1 challenge. Protection was accompanied by high levels of HA and N1 specific antibodies [70]. Notably, this recombinant is able to provide cross-protection against H5N1 and H7N1 highly pathogenic avian influenza

virus challenge (HPAI), presumably due to cross-reactive immunity conferred by the common N1 protein between these two HPAI types. This vaccine was licensed in China and over 600 million doses of this vaccines were sold by 2009 [71]. Attempts have been made to co-express cytokines along with HA protein to enhance immunogenicity of FWPV-influenza recombinants. Improvement in protective efficacy have been reported by co-expression of cytokines like chicken interleukin 2, chicken interleukin-18 along with the HA protein [72, 73].

A fowlpox vaccine recombinant expressing the H5 haemagglutinin gene provide protection against clinical signs and mortality in chicken following challenge by nine diverse highly pathogenic avain influenza viruses [68]. This vaccine overcomes limitations of many FWPV-influenza recombinants which fail to provide cross-protection to different influenza subtypes. The protection was correlated with the amino acid sequence similarity of H5 gene of challenge virus and the H5 gene inserted in the recombinant FWPV vector [68].

A recombinant FWPV expressing either Newcastle disease virus (NDV) haemagglutinin-neuraminidase (HN) or fusion (F) proteins or recombinants expressing both proteins have been developed [74–77]. Most of the recombinants use the VV7.5 or H6 early-late promoters to drive expression of HN or F gene in FWPV. Fowlpox virus recombinant vectors expressing HN and F proteins provide 100% protection to chickens against lethal velogenic NDV challenge [74, 75, 77]. One of these FWPV-based recombinant vectors, designated TROVAC-NDV, has been licensed for commercial use in the poultry industry. A single dose of TROVAC-NDV can induce high levels of hemagglutination-inhibiting antibodies in chickens for up to 8 weeks post immunization [75]. Recombinant FWPV expressing the NDV fusion (F) gene are also capable of inducing anti-F protein antibody responses which can provide protection to the chickens from lethal NDV challenge [74, 78]. The expression levels of NDV fusion (F) gene can be increased by inserting F

protein into non-essential genes in the inverted terminal repeats (ITR) of FWPV. This occurs because a foreign gene inserted into ITR region of a poxvirus vector is usually duplicated by homologous recombination, with one copy of the gene being inserted in the 5' and 3' ITR, thus increasing the expression levels of the foreign protein [74]. This strategy, however, can also lead to loss of the gene insert due to increased instability of the resultant vector. Fowlpox-NDV recombinants administered through the intramuscular or wing-web method induce stronger immune response than that of oral or ocular inoculation [77, 78]. Several fold increase in NDV HI titers is seen when chickens are primed with live or inactivated NDV vaccine and boosted with recombinant fowlpoxvirus expressing HN proteins [79].

Fowlpox vectors expressing the envelope glycoprotein of reticuloendotheliosis virus (REV) induce neutralizing antibodies and protection in chicken from viremia and runting stunting syndrome following REV challenge [80]. Synthetic promoter P_s induces higher level of expression of envelope glycoprotein than vaccinia $P_{7.5}$ promoter. Similary, recombinant FWPV expressing different genes of Marek's disease virus (MDV) have been constructed. Of these FWPV-MDV constructs, those containing one gene of MDV provide less than 50% protection in chickens. Whereas, a synergistic effect is seen when multiple genes of MDV are expressed resulting in increased protection up to 72%. Additionally, enhanced protection (94%) was seen when FWPV-MDV recombinants were given along with the MDV closely related turkey herpesvirus (HVT) [81].

Fowlpox vector expressing the VP2 protein of infectious bursal disease virus (IBDV) can protect chickens from mortality [82, 83]. However, these recombinants cannot protect chickens against damage to the bursa of Fabricious [82] and protection levels are lower than the oil adjuvanted inactivated whole virus vaccine [84]. Recombiant rFPV expressing VP2-VP4-VP3

polyprotein of IBDV inserted within TK gene under vaccinia P.L11 late promoter fails to develop protective antibodies against IBDV, whereas rFPV expressing only VP2 under fowlpox early/late promoter inserted immediately downstream of TK gene can express 5 times more VP2 protein than the former construct and also develops antibodies to IBDV [84]. Therefore, the choice of promoter and insertion site can significantly affect the immunogenicity of poxvirus vectors. In addition to viral diseases, recombinant FWPV-vectored vaccines have also been developed for non-viral diseases of poultry, including coccidiosis and mycoplasma [85, 86]. The Vectormune FP-MG, consists of a FWPV recombinant vector expressing the 40k and *mgc* genes of *M. gallisepticum*, that is licensed in the US for use in chickens and turkeys [86].

Canarypox virus-based vectors

Another avipoxvirus that has been widely used as a vaccine vector is canarypox virus (CNPV). A plaque purified clone of CNPV designated ALVAC is widely used as vector. This clone was obtained after serial passage of wild-type CNPV for 200 passages in CEF (Paoletti et al., 1998). The safety and immunogenicity profile of CNPV ALVAC vector led to its use even in human clinical trials as an HIV/AIDS vaccine candidate [88]. Additionally, there are several ALVAC-based vectored vaccines licensed for veterinary use, for example, ALVAC-AI-H5 (Influenza virus), ALVAC-RV (Rabies virus), and ALVAC-CDV-H/F(Canine distemper virus) [89].

Canarypox virus recombinants expressing the RabV G are known to elicit high levels of neutralizing antibodies in mice, cats and dogs. The level of protection observed after challenge infection was comparable to that induced by replication competent VACV vector [90]. A CNPV vector expressing haemagglutinin (HA) gene of equine influenza virus (rCNPV-EIV) induces both humoral and cellular immune responses against EIV. Cellular immune response is characterized

by increased levels of IFN-γ in vaccinated ponies. Clinical signs and virus shedding were significantly reduced in rCNPV-EIV vaccinated group after challenge infection [91]. A CNPV recombinant expressing the prM/E proteins of West Nile virus (WNV) has been licensed for use in horses [92]. A single dose of this vaccine protects horses against viremia caused by challenge with WNV-infected mosquitoes [93]. Two doses of this vaccine can provide protection for at least one year post vaccination [92, 94]. Similarly, rCNPV expressing *env* and *gag* genes of feline leukemia virus (FeLV) provides protection to cats against oro-nasal challenge with FeLV. The cats are protected from contact challenge for at least 1 year [95, 96]. The rCNPC-FeLV vaccine has been licensed for commercial use under trade name EURIFEL FeLV [95]. Both rCNPV-WNV and rCNPV-FeLV can provide protection despite absence of measurable antibody responses [93, 96]. The protection observed might be related to the activation of cell mediated immunity which requires relatively lower antigen load/dose. This phenomenon has been observed in rCPNV expressing glycoprotein (G) and fusion gene (F) of Hendra virus (HeV). The higher tested dose of rCNPV-HeV recombinant induced strong neutralizing antibodies in horses and hamsters, whereas at lower doses, partial protection was observed in hamsters despite the absence of detectable HeV specific antibodies [97]. Canarypox-virus vaccine vectors expressing glycoprotein (G) and fusion (F) gene of Nipah virus, when given in combination, can induce high levels of neutralizing antibodies in pigs and can provide solid protection from NiV challenge. In addition, vaccinated pigs show balanced Th1 and Th2 response with the induction of TNF-α, IL-10, and IFN-γ cytokines [98]. These rCNPV-HeV and rCNPV-NiV recombinants induce cross-neutralizing antibody against closely related Nipah virus (NiV) and Hendra virus (HeV) respectively.

The impact of maternal antibodies on the efficacy of avipoxvirus virus vectored vaccines has been a subject of constant debate. This is important because layers are routinely immunized

against FWPV which can impact FWPV vector-based vaccination in day old chicks. It has been demonstrated that recombinant FWPV expressing the HN gene of NDV failed to induce immune response in chickens previously vaccinated with FPV [79]. In contrast, there are reports that demonstrate that the FWPV or CNPV vectors remain effective in the presence of pre-existing immunity and can be used repeatedly without an adverse effect on the vaccine potency [61, 99, 100]. Additionally, pre-existing immunity against the heterologous gene inserted into avipox vector may interfere with the efficacy of the vaccine. For example, in the presence of maternally derived NDV antibodies the humoral response provided by FWPV-NDV recombinant expressing HN protein of NDV was dampened [75], nevertheless significant level of protection against NDV was still achieved.

Parapoxvirus as vectors

The genus *Parapoxvirus* includes four species – *Bovine popular stomatitis virus* (BPSV), *Orf virus* (ORFV), *Parpoxvirus of red deer in New Zealand* (PVNZ) and *Pseudocowpox virus* (PCPV). The type species parapoxvirus ORFV has been widely used as vector. Some of the features that make ORFV an attractive candidate vector are 1) its restricted host range (sheep and goat) 2) its ability to induce humoral and cellular immune response even in non-permissive hosts [20, 101, 102] 3) its tropism that is restricted to the skin and the absence of systemic infection 4) the fact that ORFV induces short-lived ORFV-specific immunity and does not induce neutralizing antibodies which allows multiple immunization with the same vector [103], and 5) the immunomodulatory properties of the virus [104] . In fact, inactivated ORFV is used as immunomodulator in horses and has been shown to be effective in reducing clinical signs and shedding related to equine herpes virus type 1 (EHV-1) and *Streptococcus equi* (*S. equi*) infections [105]. There are several well-characterized immunomodulatory proteins (IMPs) present in orf

virus. These IMPs include an interleukin 10 homologue (vIL-10) [106], a chemokine binding protein (CBP) (Seet et al., 2003), an inhibitor of granulocyte-monocyte colony-stimulating factor (GMC-CSF) [108], an interferon resistance gene (VIR) [109], a homologue of vascular endothelial growth factor (VEGF) [110], and at least four inhibitors of nuclear-factor kappa (NF-ƙB) signaling pathway [111–114]. Presence of these well-characterized IMPs provide a unique opportunity for rational engineering of ORFV based vectored vaccines. Two strains of ORFV, D1701 and OV-IA82, have been explored as vectors for veterinary application.

The highly attenuated D1701 strain was obtained after serial cell culture passage of an ORFV isolate from sheep in African green monkey kidney cells. This virus is apathogenic in sheep and is well adapted to grow in cell culture [115]. Adaptation of D1701 in vero cells, designated as D1701-V, led to further attenuation of virus because of additional genomic deletions. This virus is non-pathogenic even in immunosuppressed natural host sheep [116]. The utility of D1701-V strain as a vector has been extensively studied. In most constructs, the VEGF locus of D1701-V have been used to insert heterologous genes utilizing early promoter of VEGF gene (Pv). The D1701-V recombinant expressing rabies glycoprotein can stimulate high levels of rabies virus specific neutralizing antibodies in mice, cats and dogs [117]. Another recombinant expressing p40 protein of Borna virus provides protection to mice against Borna virus challenge and leads to the virus clearance from the infected brain eliminating persistent virus infection [118]. Similarly, orf virus recombinant (D1701-V) expressing haemagglutinin protein of influenza virus (H5N1) provides solid protection to mice against influenza A virus H5N1 and heterologous influenza A H1N1 challenge in a dose dependent manner [119]. Orf virus recombinant (D1701-V) expressing the major capsid protein VP1 of rabbit hemorrhagic disease virus (RHDV) protected rabbits against lethal RHDV infection with a single immunization with a dose as low as 10^5 PFU [120]. Higher

or multiple doses were required to induce significant humoral response in serum, nevertheless single dose of 10^5 PFU was enough to provide protection. This dose is significantly lower than that of VACV or canarypox recombinants expressing RHDV VP1 which require 10^7-10^9 PFU for protection when given subcutaneously, orally or intradermally [120–122].

The D1701-V has also been used as a vector in pigs. The D1701-V recombinant expressing glycoproteins gC and gD of Pesudorabies virus (PRV) induces strong cellular and humoral immune response when used to boost the pigs primed with Sindbis virus derived plasmid expressing gC and gD [123]. This type of heterologous prime-boost strategy, using DNA vaccine or baculovirus expressed protein for priming followed by boosting with ORFV based vaccine has been shown to be more effective than homologous prime-boost strategy (Rooij et al., 2010; Voigt et al., 2007). Orf virus recombinant expressing the E2 glycoprotein of classical swine fever virus (CSFV) confers solid protection against CSFV challenge in pigs [125]. A single intramuscular immunization of this recombinant induces high levels of CSFV-specific neutralizing antibodies and IFN-γ production. Interestingly, multiple site application was shown to be superior to single site injection with the same dose. This might be due to effective antigen processing and presentation occurring at different lymph nodes at the same time [125].

Another ORFV strain that has been used as a vector is OV-IA82. ORFV strain IA82 (OV-IA82) was obtained from the nasal secretion of a lamb at the Iowa Ram Test Station during an orf outbreak in 1982 and was passaged in ovine fetal turbinate cells [126]. Four genes of ORFV (ORFV002, ORFV024, ORFV121, ORFV073) that are involved in inhibition of host nuclear factor kappa (NF-κB) pathway have been well-characterized [111–114]. Deletion of ORFV121 from viral genome attenuates the virus as evidenced by decreased pathogenesis in sheep which makes ORFV121 deletion mutant an attractive vector for livestock [111]. An ORFV (OV-IA82)

recombinant expressing the full length spike (S) glycoprotein of porcine epidemic diarrhea virus (PEDV), containing a deletion of ORFV121 gene induces PEDV-specific neutralizing antibodies in pigs and protects pigs from clinical signs of PED [20]. This ORFV-PEDV-S recombinant was also shown to induce passive immunity and transfer of PEDV-specific IgG, IgA and neutralizing antibody to piglets via milk and colostrum. Upon challenge with virulent PEDV, there is decreased clinical signs and reduced mortality in piglets born from ORFV-PEDV vaccinated sows. Additionally, increased protection with 100% survival was obtained when sows were primed with live PEDV and then boosted with the ORFV-PEDV-S recombinant [127].

In addition to ORFV121, another NF-κB inhibitor, ORF024, has also been used as a site for foreign gene insertion. The immunogenicity of two ORFV-IA82 recombinants constructed by inserting RABV G in the ORFV121 or ORFV024 gene loci were evaluated in pigs and cattle [101]. Both recombinants induced robust neutralizing antibody response against RABV in pigs and cattle. Notably, the neutralizing antibody titers induced by ORFV121 deletion mutant were higher than that of ORFV024 deletion mutant [101]. This type of differential regulation of innate and adaptive immune response has also been reported for NF-κB inhibitor proteins encoded by vaccinia virus (VACV), where deletion of one NF-κB inhibitor (A52R) leads to higher immune response against heterologous antigen when compared to other NF-κB inhibitors (B15,K7) [128].

Despite restricted host range, infections of humans have been reported for parapoxviruses. The virus causes self-limiting infection that usually involved hands of the people. Immunocompromised people and farmers who enter in close contact with infected animals and -present abrasions or cuts in ths skin can become infected with ORFV [129–131]. Since, the recombinant ORFV that have been used as vector platforms (D1701 and OV-IA82Δ121) are known to be non-pathogenic in sheep and goat, it is safe to assume that these recombinants should

not be able to cause human infections.

Swinepox virus as vector

Swinepox virus (SPV) is the only member of genus *Suipoxvirus*. SPV causes mild self-limiting infection in pigs. Because of its narrow host range restricted to pigs, SPV-based recombinant vectored vaccine candidates have been mostly developed for use in pigs. One of the first attempts to use SPV as vector involved a recombinant SPV targeting Aujeszky's disease (Psaudorabies virus; PRV) [132]. The gp40 and gp63 genes of PRV were inserted into TK locus of SPV under the early/late $VV_{7.5}$ promoter. At 21 days post-immunization, 90% of the pigs vaccinated by scarification developed serum neutralizing antibody against pseudorabies virus, whereas 100% of the animals vaccinated by the intramuscular route developed neutralizing antibodies. Significant level of protection was observed in pigs upon challenge with virulent PRV [132]. A recombinant SPV expressing the E2 glycoprotein of classical swine fever virus (CSFV) in the TK locus expresss the E2 protein in a dimeric form in the cytoplasm of the infected cells [133]. However, this recombinant was not tested in animal model. SPV recombinant expressing HA1 gene of swine influenza (SIV; H1N1) elicits humoral and cellular immune responses and provides complete protection against SIV in swine and mice [134]. Although neutralizing antibody titers were low (1:8 to 1:32), potent Th1 and Th2 responses were observed as evidenced by increased levels of IL-4 and IFN-γ, which may have contributed to protection against SIV challenge. Co-expression of the HA gene of H1N1 and H3N2 subtype provide complete protection against H1N1 and H3N2 challenge in pigs [135].

Swinepox virus recombinants expressing immunodominant epitopes of certain viral antigens have also been shown effective. A recombinant SPV expressing the A epitope of transmissible gastroenteritis virus (TGEV) spike protein induced neutralizing antibodies and

strong Th1 and Th2 cytokine responses against TGEV. Notably, when neutralizing antibodies purified from vaccinated animals were fed to piglets, they were protected against severe disease and mortality after challenge with TGEV [136]. Similarly, a recombinant SPV expressing three repeats of a conserved six-amino acid epitope present in the N-terminal ectodomain of the GP3 protein of porcine reproductive and respiratory syndrome virus (PRRSV) induced cellular and humoral response in pigs [137]. Swinepox virus has also been used to develop recombinant vaccine candidates against bacterial diseases. A recombinant swinepox virus expressing M-like protein (SzP) of *Streptococcus equi spp. zooepidemicus* (SEZ) provided significant protection against SEZ infection [138].

Swine is the major reservoir of SPV, and the virus does not infect several mammalian and avian species. Initially, SPV was known to infect only cells of porcine origin, however, recent findings have shown that SPV exhibit a relatively broad cell culture host range *in vitro* [139, 140]. Recombinant SPV can infect, present limited replication and express foreign genes in cells of non-porcine origin including human, monkey, hamster and rabbit cell lines [140]. Moreover, when inoculated intradermally, SPV causes productive infection in rabbits [141]. These findings have opened opportunities of using SPV vector in different animal species. A SPV recombinant expressing the *gag* and *env* proteins of feline leukemia virus (FeLV) fails to replicate in feline cells, but FeLV gag virus-like particles were produced in feline cells and incorporated into SPV intracellular mature virion (IMV) [142]. The immunogenicity of this recombinant virus, however, was not tested in cats.

Deletion of the TK gene has been widely used in recombinant poxvirus vector construction. However, deletion of the TK gene may not be the best strategy to generate SPV-based recombinant vectors as it results in severe attenuation of the vector and consequently in lower vaccine

efficiency. It has been shown that deletion of the TK gene results in decreased neutralizing antibody levels and in Th1 and Th2 mediated immune responses [143]. The ability of the virus to replicate in non-permissive cells also decreases significantly in TK deletion mutants [143]. Insertion of foreign antigens in intergenic or non-coding regions of the SPV genome could be used as an alternative approach. A SPV recombinant expressing the porcine IL-18, the capsid protein of porcine circovirus 2, and the SzP protein of SEZ in the intergenic region between SPV020 and SPV021 open reading frames induces immune responses against both PCV2 and SEZ, which was comparable to the immune responses elicited by commercial vaccines [144]. These findings suggest that SPV can be used as a vector platform for multivalent vaccines against diseases of swine.

Capripox virus as vector

The genus *Capripoxvirus* includes *Goatpox virus* (GTPV), *Sheeppox virus* (SPPV) and *Lumpy skin disease virus* (LSDV). SPPV and GTPV infect sheep and goats respectively, with some isolates being able to infect both species. LSDV causes disease in cattle and buffalo [145]. These three species of CPV share 96-97% nucleotide identity [146, 147]. Because of the high degree of sequence conservation, cross-immunity is observed among the three viruses. An attenuated LSDV deficient of an IL-10 gene homologue (ORF005) has been shown to provide protective immunity against virulent capripoxvirus challenge in sheep and goats [148]. Thus, theoretically, an attenuated strain of any capripoxvirus should be able to protect against SPPV, GTPV and LSDV [149]. Using these viruses as vectors, it is possible to generate multivalent vaccines against CPV and other target pathogens of ruminants. Moreover, the replication of capripox viruses is restricted to ruminants with no evidence of human infections. These traits make CPVs good candidates for developing recombinant vectored vaccines.

Capripox viruses have been mainly used to develop recombinant vaccines for use in ruminants. Most of the recombinants have been generated using Kenya-strain-1 (KS-1), which was isolated from sheep and passaged in lamb testis- and baby hamster kidney (BHK) cells [150]. Recent molecular studies have shown a close relationship between KS-1 and LSDV, suggesting KS-1 may actually be LSDV [146]. The majority of KS-1 based constructs have been generated by inserting the foreign gene into the TK locus. Two individual KS-1 recombinants generated by insertion of the fusion (F) protein or hemagglutinin (H) protein of rinderpest virus (RPV) into TK gene locus under the control of vaccinia virus late promoter P11 protect cattle against rinderpest virus after lethal challenge with a virulent RPV isolate. The recombinant with the H gene insert showed better protective efficacy than the vector expressing the F gene. Both recombinants protected cattle against LSDV challenge in addition to rinderpest [151, 152]. Interestingly, these recombinants protect goats from lethal challenge with peste des petits virus (PPRV) challenge [153]. Another recombinant capripoxvirus expressing the H and F gene of PPRV has been developed using AV41 strain of GTPV. A single dose of this recombinant elicits seroconversion in ~80% of immunized sheep and goats. Neutralizing antibodies are detected up to 6 months after vaccination. Two doses of this vaccine completely overcome the interference caused by pre-existing immunity to caprinepox virus [154]. Another recombinant CPV expressing both H and F genes of PPRV confers an earlier and stronger immune response against PPR and GTPV [155].

A KS-1 recombinant expressing the VP7 protein of blue tongue virus (BTV) provides partial protection in sheep [156]. Partial protection against BTV was achieved even when sheep were immunized with the combination of KS-1 recombinants individually expressing four proteins (NS1, NS3,VP2, VP7) of BTV [157]. Capripoxvirus has been used to develop recombinant vaccine candidates against Rift valley fever virus (RVFV) [158, 159]. A recombinant KS-1 virus

expressing Gn and Gc glycoproteins of RVFV induced neutralizing antibodies to RVFV in sheep and two doses of the vaccine candidate provided significant protection against RVFV and SPPV challenge in sheep.

In addition to KS-1 strain, an attenuated strain of LSDV, Neethling strain, has also been used as vector. This strain is used as vaccine against Lumpy skin disease in Africa. The ribonucleotide reductase gene of Neethling strain has been identified as potential insertion site for recombinant vector generation [160]. The Neethling strain has been used to develop recombinant vaccines against RabV, bovine ephemeral fever and RVFV viruses [145]. Recombinant LSDV (Neethling strain) generated by inserting RabV G in the ribonucleotide reductase gene locus induced strong cellular and humoral response in cattle [160]. Neutralizing antibody titers as high as 1513 IU/mL were observed in cattle. This recombinant also induced robust humoral and cellular immunity in non-permissive hosts (mice and rabbits). Immunization of mice with this recombinant vector protected the animals from an aggressive intracranial RabV challenge [161].

Because of the cross-protection offered by CPVs, theoretically it should be possible to develop a universal recombinant CPV vector that would provide protection against all capripox viruses in addition to target pathogen. However, the geographical distribution of the different CPV limits that possibility. Sheep pox and goat pox viruses are endemic to Asia, Middle East and Africa south of the equator, whereas LSDV is mainly present in sub-Saharan Africa [145]. A country would refuse to use capripoxvirus vectored vaccine if the vector is not endemic. Future research should aim at identifying immunomodulatory genes and virulence factors encoded by CPV, which would allow the development of safer recombinant CPV-based vectors that could potentially be used in both endemic and non-endemic countries.

Leporipoxvirus as vector

Myxoma virus (MYXV), the type species of genus *Leporipoxvirus*, specifically infects rabbits and hare (leporides). MYXV was used as biological agent to control European rabbit population in Australia. This method, however, was not sustainable because of the coevolution of the virus and the rabbit which led to the adaptation of the virus to the novel host species [162].

MYXV has been used as vector for both leporide and non-leporide species. A recombinant myxoma virus expressing capsid protein (VP60) of rabbit hemorrhagic disease virus (RHDV) has been shown to protect rabbit from myxomatosis and RHDV [122]. MYXV expressing haemagglutinin (HA) of influenza virus can induce high levels of anti-HA antibodies in rabbits as efficiently as VV vector [163]. MYXV recombinant has been shown to be effective in protecting cats from feline calicivirus [164]. The possibility of using MYXV as a non-replicative vector in small ruminants (sheep) has been demostrated [165]. Moreover, the use of MYXV-vectored immunocontraception as a means to control wildlife species have been assessed [166]. Myxoma virus expressing immunocontraceptive antigen (zone pellucida 3 [rZp3] glycoprotein of rabbit) was able to induce autoimmune infertility in 70% of rabbits at the first breeding [167].

MYXV encodes several proteins that are known to cause immunosuppression in rabbits [168]. Deletion of those immunosuppressive genes is essential to enhance the safety and immunogenicity of the MYXV vector.

Future directions and potential for other applications

Poxvirus-based vectors have long been used as vaccine delivery platforms in many animal species. The overall immunogenicity, safety and broad disease and species applicability of these viral vectors make them especially attractive for vaccine delivery in veterinary medicine. After decades of research, there is a general consensus that replication-competent poxvirus vectors can induce better immune responses in target species, but they have potential for infecting non-target

hosts (e.g. vaccinia virus). In contrast, replication-deficient poxviruses are safer, but induce comparatively lower immune responses. Hence, future studies focusing on rationally designed poxvirus vectors platforms could lead to more balanced vectors, with an improved safety profile and immunogenicity. Poxviruses are known for encoding several immunomodulatory proteins (IMPs) that target host immune responses to allow efficient virus infection and replication. Most importantly, several of these genes encode for virulence determinants that contribute to poxvirus disease pathogenesis. By targeting those genes, one could expect to attenuate a given poxvirus vector and, perhaps, simultaneously enhance its immunogenicity in target animal species. As additional poxviral IMPs are identified and characterized, we are likely to see the development and refinement of poxviral vectors.

In addition to modulation of poxvirus vector safety and immunogenicity, the field would greatly benefit from additional studies focused on identifying better promoters for expression of heterologous genes. Promoters that would allow sustained gene expression following immunization would likely result in more robust and long-lasting immune responses. There is also a need for better recombinant selection methods. The use of CRISPR/Cas-9 in combination with fluorescent-activated cell sorting is a promising approach that may facilitate and speed up the selection process. Additional research assessing the effect of dose and route of immunization, stability of the recombinants, sustained heterologous gene expression and perhaps even the use of host cytokines to enhance T-cell responses, will be pivotal in developing safe and immunogenic

Vaccine Trade Name*	Target pathogen	Target species	Insert gene	Poxvirus vector	Reference
RABORAL V-RG	Rabies	Wildlife canines	Glycoprotein	Vaccinia virus	[40, 169]

ProteqFlu	Equine influenza	Horses	HA	Canarypox	[170, 171]
RecombiTek Equine Influenza	Equine influenza	Horses	HA	Canarypox	[170, 171]
Recombitek West Nile Virus	West Nile Virus	Horses	preM/E	Canarypox	[93, 170]
PUREVAX Feline Rabies	Rabies	Cats	Glycoprotein	Canarypox	[172]
PUREVAX Recombinant FeLV	Feline leukemia virus	Cats	Env,Gag/pol	Canarypox	[95, 172]
Recombitek Distemper	Canine distemper virus	Dogs	HA and F	Canarypox	[172, 173]
PUREVAX Ferret Distemper	Canine distemper virus	Ferrets	HA and F	Canarypox	[172]
Vectormune FP MG	Mycoplasma gallisepticum	Poultry	40 k and mgc	Fowlpox	[86]
Vectormune FP LT	Laryngotracheitis	Poultry		Fowlpox	[174]
Vectormune FP-ND	Newcastle diseae virus	Poultry	HN and F	Fowlpox	[75, 172]
TROVAC-AIV-H5	Avian influenza	Poultry	HA	Fowlpox	[67]

poxvirus-based vectored vaccines.

Table 1. Licensed and commercially available poxvirus-vectored vaccines

*Trade name might differ according to country and manufacturer.

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CHAPTER 2

Passive immunity to porcine epidemic diarrhea virus following immunization of pregnant gilts with a recombinant orf virus vector expressing the spike protein

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Passive immunity to porcine epidemic diarrhea virus following immunization of pregnant gilts with a recombinant orf virus vector expressing the spike protein

Abstract

Passive immunity is critical for protection of neonatal piglets against porcine epidemic diarrhea virus (PEDV). Here, we investigated the immunogenicity of an orf virus (ORFV) vector expressing the full-length spike (S) protein of PEDV (ORFV- PEDV-S) in pregnant gilts and its ability to confer passive immunity and protection in piglets. Three doses of ORFV-PEDV-S were given to two groups of PEDV-negative pregnant gilts, with the last dose being administered two weeks prior to farrowing. One of the two groups immunized with the ORFV-PEDV-S recombinant virus was also exposed to live PEDV orally on day 31 post-immunization (pi). Antibody responses were assessed in serum, colostrum and milk of immunized gilts, and passive transfer of antibodies was evaluated in piglet sera. The protective efficacy of ORFV-PEDV-S was evaluated after challenge of the piglets with PEDV. PEDV-specific IgG, IgA and neutralizing antibody (NA) responses were detected in ORFV-PEDV-S-immunized and ORFV-PEDV-S-immunized/PEDV-exposed gilts. PEDV NA, IgG and IgA were detected in the serum of piglets born to immunized gilts, demonstrating the transfer of antibodies through colostrum and milk. Piglets born to immunized gilts showed reduced morbidity and a marked reduction in mortality after PEDV challenge in comparison to control piglets. Piglets born to gilts that received ORFV-PEDV-S and were exposed to live PEDV showed stronger NA responses and lower clinical scores when compared to piglets born to gilts immunized with ORFV-PEDV-S alone. These results demonstrate the potential of ORFV as a vaccine delivery platform capable of eliciting passive immunity against PEDV.

1. Introduction

Porcine epidemic diarrhea virus (PEDV), an alphacoronavirus in the family *Coronaviridae*, is a single-stranded, positive-sense RNA virus that causes porcine epidemic diarrhea (PED) in pigs [1]. PEDV infects pigs of all age groups resulting in an enteric disease with high morbidity. High mortality rates (50%-100%) are observed in neonatal piglets; however, mortality is usually low in older animals [2]. PED is characterized by vomiting, watery diarrhea and dehydration, which are usually followed by death in suck- ling piglets [3]. After its introduction in the USA in 2013, PEDV caused the deaths of over 7 million piglets, resulting in significant economic losses to the US swine industry [4]. Since then, significant investments and efforts to develop PEDV vaccines have been made, with a few vaccines receiving a conditional license from the USDA. The efficacy of these vaccines in protecting newborn piglets, however, is still unknown.

Piglets are born agammaglobulinemic due to the impermeable nature of the epitheliochorial swine placenta, and their immune system is immature, which makes them highly susceptible to PEDV in the first weeks (1-3) of life. Therefore, passive transfer of antibodies through colostrum and milk is critical for protection of neonatal piglets against PEDV [5, 6]. Previous studies with transmissible gastroenteritis virus (TGEV), a coronavirus that is closely related to PEDV, have shown a high correlation between milk antibody levels and protection in piglets [7, 8]. Hence, a requirement for an effective PEDV vaccine is the ability to induce high levels of antibodies in colostrum and milk with their subsequent transfer to piglets born to immunized sows. Although several attenuated, killed, or subunit PEDV vaccines have been developed, most of them fail to induce sufficient levels of lactogenic immunity and protection in newborn piglets [9, 10]. Thus, it is critical to develop improved alternatives to currently available PEDV vaccines.

Recently, we demonstrated that a recombinant orf virus (ORFV) expressing the full-length spike (S) protein of PEDV (ORFV-PEDV-S) is capable of eliciting protective immune responses in immunized pigs [11]. Three-week-oldpiglets immunized intramuscularly (IM) with the ORFV-PEDV-S recombinant virus were protected from clinical signs of PED after oral challenge with PEDV and showed reduced virus shedding in feces [11]. In the present study, weinvestigated the immunogenicity of ORFV-PEDV-S recombinant virus in pregnant gilts and its ability to induce passive immunity and protection in piglets born to immunized animals.

Materials and methods

Cells and viruses

The recombinant ORFV-PEDV-S was previously generated and characterized in our laboratory [11], propagated and titrated in primary ovine fetal turbinate cells (OFTu, provided by D.L. Rock, University of Illinois) [11]. PEDV strain USA/CO/2013 (CO13) was obtained from the National Veterinary Services Laboratory (NVSL) and propagated in Vero-76 cells (ATCC® CRL-1587™) in the presence of 1.5 μg of TPCK-treated trypsin per mL (Sigma Aldrich, St. Louis, MO).

Immunization of pregnant gilts and challenge studies

Six primiparous gilts were randomly assigned to three experimental groups as follows: group 1 (G1), control (MEM; n = 2); group 2 (G2), ORFV-PEDV-S-immunized (n = 2); group 3 (G3), ORFV-PEDV-S-immunized/live PEDV-exposed (n = 2) (Table 1). Animals from G2 and G3 were immunized intramuscularly (IM) with 2 ml of the recombinant ORFV-PEDV-S containing 107.38 tissue culture infectious dose 50 (TCID50)/mL in MEM. All animals received the first immunization on day 0 and two booster immunizations on days 21 and 42 post-

immunization (pi). The second booster was administered two weeks prior to the anticipated parturition date. In addition to the immunization regimen with ORFV-PEDV-S described above, gilts in G3 were exposed to live PEDV orally (1 × 105 TCID50 in 1 ml) on day 31 pi. Animals from G1 were sham-immunized with 2 ml MEM as described above. Animals were housed in BSL-2 animal rooms, and two weeks prior to farrowing, each gilt was transferred to individual farrowing crates (two crates per room).

Group (n)	Treatment	Day of immunization (route)	Dose $TCID_{50}$	No of piglets
Group 1 (G1) (n = 2)	Control (MEM)	0, 21, 42 (IM)	$2 \times 10^{7.38}$	24
Group 2 (G2) (n = 2)	ORFV-PEDV-S	0, 21, 42 (IM)	$2 \times 10^{7.38}$	24
Group 3 (G3) (n = 2)	ORFV-PEDV-S Live PEDV	0, 21, 42 (IM) 31 (oral)	$2 \times 10^{7.38}$	24

Table 2.1. Experimental design

All gilts received prostaglandin-F2α (10 mg) on day 52 pi by IM injection to induce parturition. Animals from G1 and G3 farrowed on day 53 pi, while animals from G2 farrowed on day 56 pi. Twelve piglets from each sow were randomly selected, kept with their mothers (n = 24 per group) for 17 days and allowed to suckle colostrum and milk ad libitum. Excess piglets were euthanized to keep all sows with an equal number of piglets (n = 12). All piglets (G1, G2 and G3) were challenged orally on day 7 post-birth with a virus suspension containing 2.5 × 102 TCID50 of PEDV strain CO13 (1 ml/piglet). Animals were monitored daily for clinical signs and mortality, and the experiment was terminated on day 74 pi or day 11 post-challenge (pc). Clinical scores were evaluated based in four criteria, which were modified from a scoring

method described by Lohse and collaborators [12] as follows: a) well-being: normal = 0, slightly depressed = 1, depressed and lethargic = 2; b) defecation: normal feces = 0, semi-solid and pasty = 1, watery feces = 2;c) vomiting: no = 0, yes = 1; d) body condition: normal = 0, thin = 1, emaciated = 2. Mean daily group scores and mortality rates were calculated and compared between differentgroups. Piglets showing severe dehydration and emaciationwere euthanized based on the independent evaluation of SDSU's veterinarian.

Serum was collected from sows on days 0, 21, 28, 35,42, 49, 54, 60, 63, 67, 70 and 74 pi. Additionally, colostrum and milk were collected from sows on days 1 (day of farrowing) 3, 7, 10, 14, 17 post-farrowing. Serum and rectal swabs were collected from piglets on days 1 (pre-colostrum), 7 (pre-challenge), 10, 14, and 17 post-birth. All animal studies were conducted at the SDSU Animal Resource Wing (ARW), following the guidelines and protocols approved by the SDSU Institutional Animal Care and Use Committee (IACUC approval no. 16-003A).

Antibody isotype ELISA

Indirect ELISAs using a truncated S protein (aa 630-800) were used to assess IgG and IgA antibody responses in animals immunized with the recombinant ORFV-PEDV-S virus as described previously [11].

Fluorescent focus neutralization assay

Neutralizing antibody responses elicited by immunization with the recombinant ORFV-PEDV-S were assessed by fluorescent focus neutralization assay (FFN) as described previously [13].

Fluorescent microsphere immunoassay (FMIA)

PEDV S-specific IgG and IgA antibody responses were assessed in colostrum and milk by FMIA. Optimal assay conditions (amount of antigen, colostrum/milk and second- ary

antibody dilutions) were determined by a checkerboard titration [13].

Real time reverse transcriptase PCR (RT-qPCR)

Virus shedding was assessed in rectal swabs by RT-qPCR. Viral RNA was extracted from rectal swabs using a Zymo Viral RNA Extraction Kit (Zymo Research, CA, USA) according to the manufacturer's instructions. Primers and a probe targeting the PEDV nucleocapsid protein were designed using PrimerQuest Tool (Integrated DNA Technologies Inc., USA). RT-qPCR was performed using a SensiFast™ Probe Lo-ROX One-Step Kit (Bioline, MA, USA) following the manufacturer's instructions. Genome copy numbers per ml were determined using a relative standard curve method. The amount of viral RNA detected in feces was expressed as log10 genome copies/ml.

Results

Systemic antibody response in gilts

Gilts from both immunized groups (G2 and G3) developed PEDV S-specific IgG and IgA responses (Fig. 2.1Aand 2.1B). IgG and IgA antibodies were first detected on day 21 pi in animals in G2, and the highest level of antibodies was detected on day 35 pi. Similarly, in G3, IgG and IgA antibodies were first detected on day 21 pi, and their levels increased after the booster immunization on day21. An anamnestic antibody response was observed in G3 gilts after they were exposed to live PEDV on day 31 pi (Fig. 2.1A and 2.1B). No spike-specific IgG or IgA antibodies were detected in the serum of control sows (G1).

The ability of ORFV-PEDV-S to induce neutralizingantibody (NA) responses against PEDV was assessed using a FFN assay. NA were first detected in serum a weekafter the first booster immunization (day 28 pi; Fig. 2 . 1C). An increase in NA levels was observed in both immunized groups (G2 and G3) after day 28 pi (Fig. 2.1C). Gilts in G2, which were

immunized only with ORFV-PEDV-S, had the highest neutralizing antibody titers on day 49 pi, a week after the second booster immunization (day 42 pi; Fig. 2.1C), whereas gilts in G3, which were immunized with ORFV-PEDV-S and exposed to live PEDV (day 31 pi),had the highest antibody titers on day 35 pi, with titers remaining constant thereafter until the end of the experiment (Fig. 2.1C). Similar to the IgG and IgA responses,higher neutralizing antibody responses were observed in gilts in G3 when compared to G2 animals. No neutralizing antibodies against PEDV were detected in control gilts(G1) in serum samples collected pre-farrowing/pre-challenge. Animals in G1 seroconverted to PEDV, presenting detectable levels of IgG, IgA and NA a week after challenge of the piglets (day 7 post-birth; Fig. 2.1). Notably, a strong correlation between NA and IgG and IgA levels wasobserved in sow serum (NA vs. IgG: $r = 0.876$, $P < 0.0001$;NA vs. IgA: $r = 0.822$, $P < 0.0001$; Fig. 2.1D and 2.1E). These results confirmed our previous findings demonstrating the immunogenicity of ORFV-PEDV-S in pigs [11].

Detection of S-specific IgG and IgA antibodies in milk and colostrum

High levels of S-specific IgG antibodies were detected on days 1 and 3 post farrowing, decreasing thereafter in animals from G2 and G3 (Fig. 2.2A). Colostrum collected from sows in G3, exposed to live PEDV, had higher levels of IgG when compared to G2 on day 1 post-farrowing (Fig. 2.2A). High levels of S-specific IgA antibodies were also detected in colostrum of animals from G2 and G3 on day 1 post-farrowing (Fig. 2.2B). Notably, the levels of S-specific IgA antibodies remained elevated in milk up to day 17 post-farrowing (Fig. 2. 2B). IgA antibody levels were higher in milk from animals in G3 when compared to animals in G2 after day 7 post-farrowing (Fig. 2. 2B). No spike-specific IgG or IgA antibodies were detected in colostrum/milk collected from control sows (G1) (Fig. 2.2A and 2.2B). These results demonstrate the ability of

ORFV-PEDV-S to induce S-specific IgG and IgA antibodies in colostrum and milk.

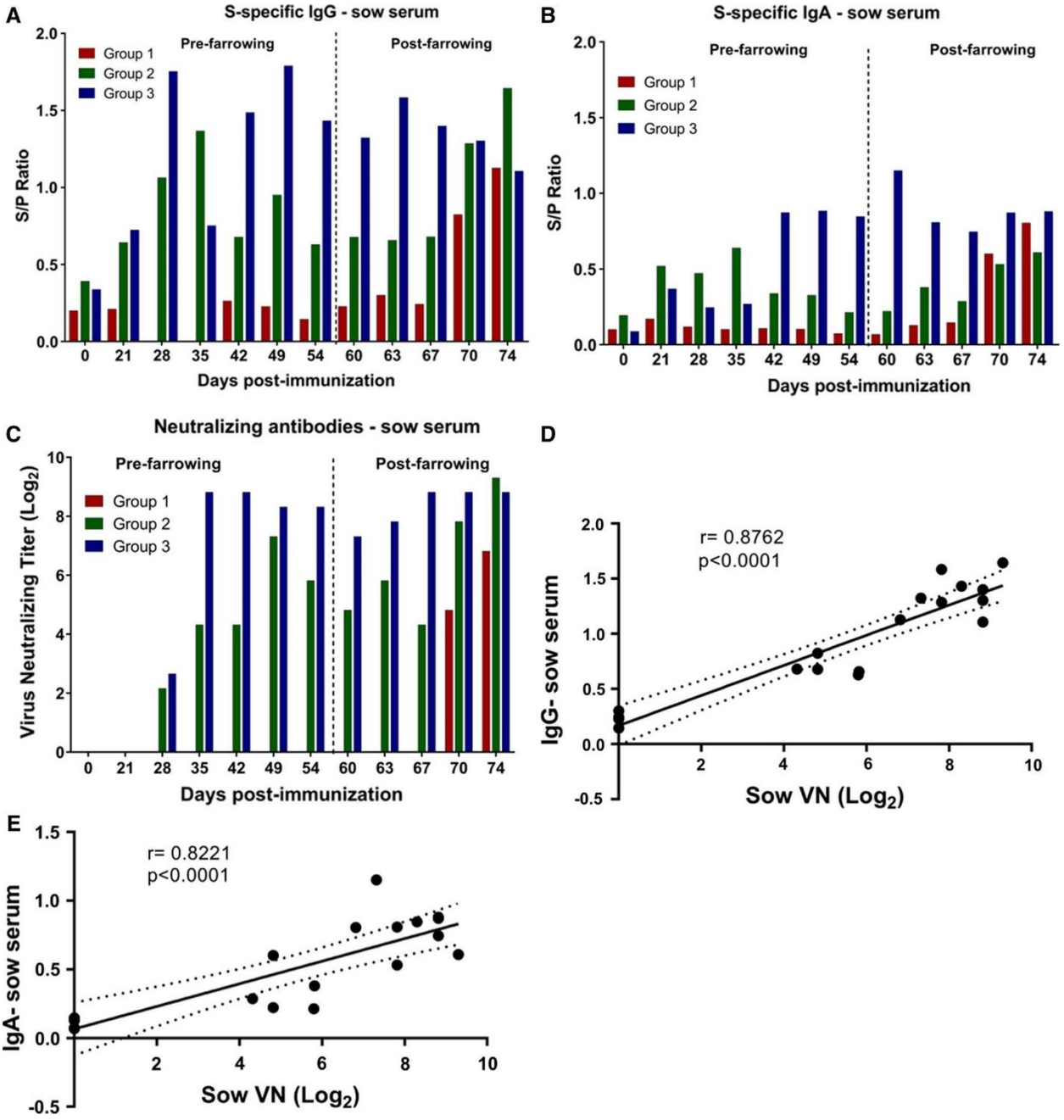

Figure 2.1 Spike (S)-specific antibody responses in sows. (A) Isotype ELISA demonstrating serum IgG antibody responses specific for the PEDV S protein. (B) Isotype ELISA demonstrating serum IgA antibody responses specific for the PEDV S protein. (C) Virus neutralizing antibody (NA) responses in sows. S/P, sample-to-positive ratio. Group 2 and group 3 gilts were immunized on days 0, 21 and 42 with ORFV-PEDV-S. Oral exposure to live PEDV in group 3 gilts was performed on day 31 pi. Group 1 control gilts were sham-immunized with

minimal essential medium (MEM) on the same days. (D) Correlation of sow serum IgG levels with sow mean NA levels. (E) Correlation of sow serum IgA levels with piglet serum NA levels. Correlations were calculated using the Spearman method with a 95% confidence interval using GraphPad Prism 7

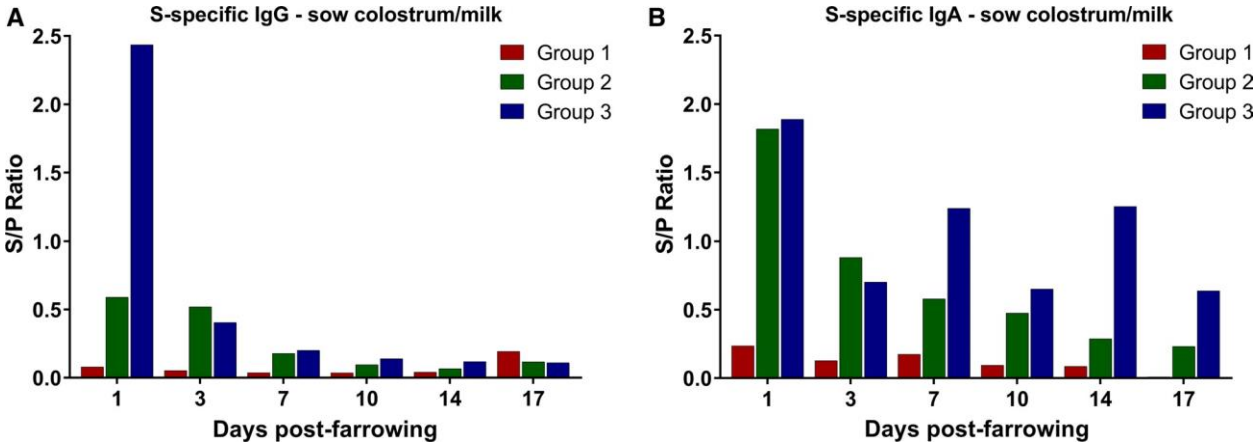

Figure 2.2 Detection of PEDV S-specific antibodies in colostrum/milk. (A) Isotype fluorescent microsphere immunoassay (FMIA) demonstrating S-specific IgG antibodies in colostrum/milk of immunized or control gilts. (B) Isotype FMIA demonstrating S-specific IgA antibodies in colostrum/milk of immunized or control gilts. S/P, sample-to-positive ratio.

Passive transfer of PEDV-specific antibodies to the piglets

Passive transfer of antibodies from immunized sows to their offspring was assessed by ELISA and FFN assays performed on serum samples collected from piglets pre- and post-ingestion of colostrum/milk. No S-specific antibodies were detected in serum samples collected on day one prior to ingestion of colostrum (Fig. 2.3). Notably, high levels of S-specific IgG and IgA antibodies were detected in the serum of piglets from G2 and G3 on day 3 post-birth. Piglets born to immunized sows had IgG and IgA antibodies in their serum until the end of the experiment on day 17 post-birth (Fig. 2.3), with higher levels of IgG antibodies being detected until the end of the experiment. Both IgG and IgA levels were higher in piglets born to sows in G3 when compared to piglets born to sows in G2. No spike-specific IgG or IgA was detected in serum of piglets born to control sows (G1; Fig. 2.3).

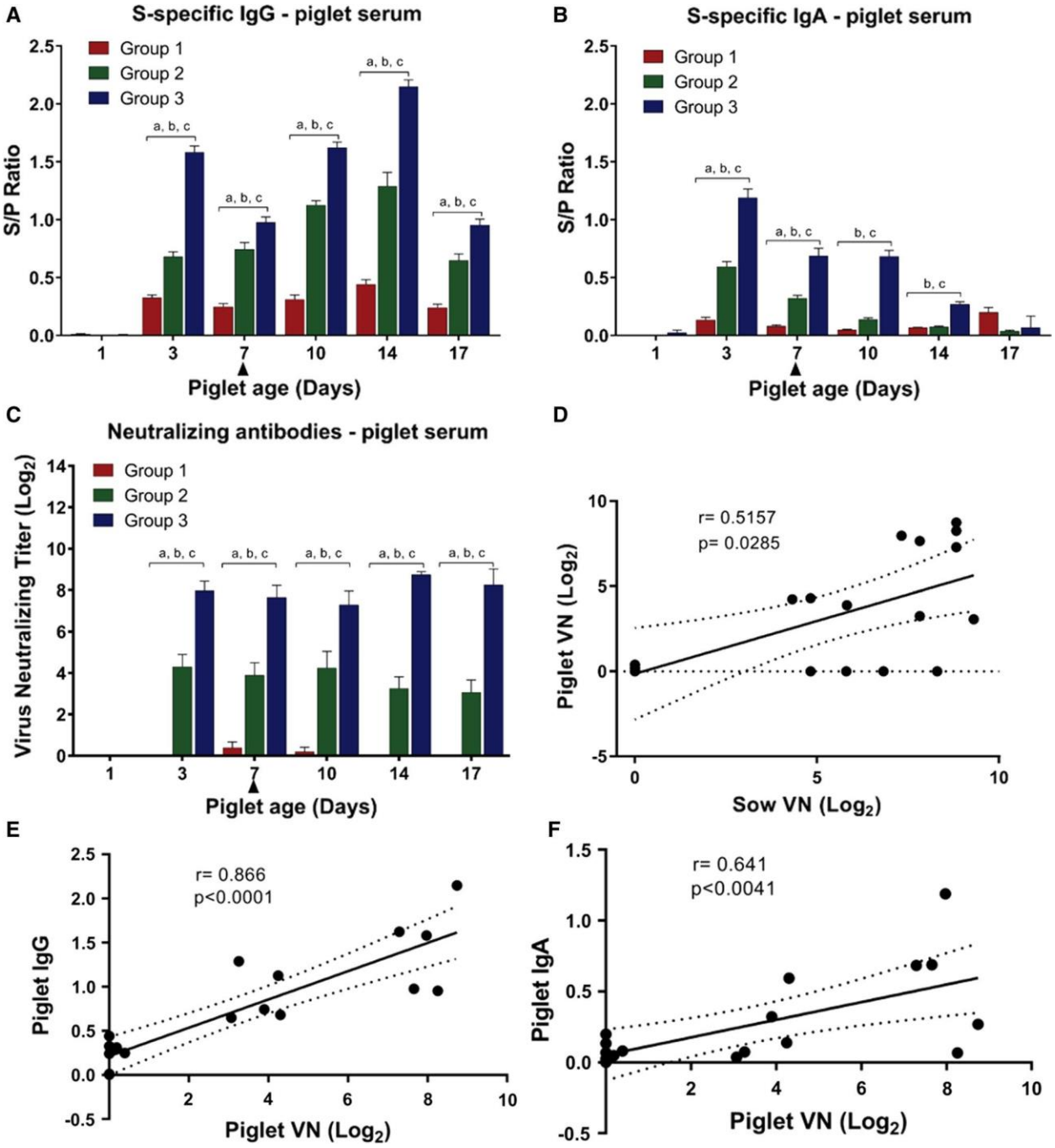

Figure 2.3. Passive transfer of antibody to piglets. (A) Isotype ELISA demonstrating S-specific IgG antibody levels in piglet serum. (B) Isotype ELISA demonstrating S-specific IgA antibody levels in piglet serum. (C) Virus neutralizing antibody (NA) levels in piglet serum. Results are presented as group mean S/P ratios or NA titers. The error bars represent +/- standard error of mean (SEM). Statistical significance was determined using two-way ANOVA, and multiple comparisons were ran using Tukey's test. a, b, and c represent statistical significance for G1 vs. G2, G1 vs. G3, and G2 vs. G3, respectively. The significance level is < 0.01. S/P, sample-to-positive ratio; the arrowhead represents the day of challenge with PEDV. (D) Correlation of piglet serum NA levels with sow mean NA levels. (E) Correlation of group mean IgG antibodies

in piglet serum with piglet serum NA levels. (F) Correlation of group mean IgA antibodies in piglet serum with piglet serum NA levels. Correlations were calculated using the Spearman method with a 95% confidence interval using GraphPad Prism 7.

In addition to IgG and IgA, NAs were detected in piglet serum on day 3 post-birth. High levels of NA were detected in piglets born to ORFV-PEDV-S-immunized sows until the end of the experiment on day 17 pi (Fig. 2.3C). No neutralizing antibodies were detected in the piglets born to control sows (G1, Fig. 2.3C). Notably, a significant correlation between NA titers in sow serum and piglet serum was observed ($r = 0.5157$, $P = 0.0285$). Additionally, a strong correlation between NA and IgG and IgA antibody levels was observed in piglet serum (NA vs. IgG: $r = 0.866$, $P < 0.0001$; $r = 0.641$; Fig. 2.3E and F). Together, these results demonstrate passive transfer of PEDV-specific IgG, IgA and NAs from immunized sows to piglets through ingestion of colostrum and/or milk.

Protection of piglets against PEDV challenge

All piglets born to immunized or control gilts were challenged orally with virulent PEDV strain CO13 on day 7 post-birth. Piglets were monitored daily for characteristic clinical signs of PED and mortality. Daily average clinical scores were calculated for each group, and the mean daily scores are presented in Fig. 2.4A. All piglets born to sows in G1 and G2 showed clinical signs of PED starting on day 1 pc which lasted until day 9-10 pc. The highest clinical scores were observed in G1 piglets between days 2 and 5 pc. Piglets in G3 started showing clinical signs on day 3 pc, when moderate diarrhea was observed and continued until day 11 pc. As shown in Fig. 2.4A, more-severe clinical signs were observed in piglets from G1 (average daily scores ranging from 4 to 7), followed by G2 (average daily scores ranging from 4 to 6) and then G3 (average daily scores ranging from 2 to 3.5). It is important to note that, while piglets in G1 and G2 experienced vomiting and diarrhea, none of the piglets in Group 3 experienced vomiting

during experiment.

Virus shedding in feces was assessed by RT-qPCR using rectal swabs. Piglets in G3 had a significantly lower level of viral RNA in their feces ($P < 0.0001$) in comparison to piglets in G1 and G2 on day 3 pc (Fig. 2.4B). However, no significant differences in virus shedding in feces were observed between the three groups thereafter.

The daily mortality recorded for piglets in the three groups is presented in Fig. 2.4C. Notably, twelve out of 24 (12/24; 50%) piglets in control G1 died by day 7 pc, while only one piglet (1/24; 5%) in G2 died on day 5 pc, and none of the piglets (0/24; 0%) in G3 died after the PEDV challenge. Interestingly, when the correlation between antibody levels in piglets and sows was compared to survival rates in piglets, moderate to strong correlations between NA levels in piglet serum ($r = 0.4819$, $P = 0.04288$) and IgA and IgG antibody levels in the sow colostrum/milk ($r = 0.8759$, $P < 0.0001$; $r = 0.6706$, $P = 0.002322$, respectively) were observed (Fig. 2.4D, E and F). Together, these results demonstrate decreased morbidity and mortality in piglets born to immunized gilts and a strong correlation between survival and antibody levels in colostrum and milk.

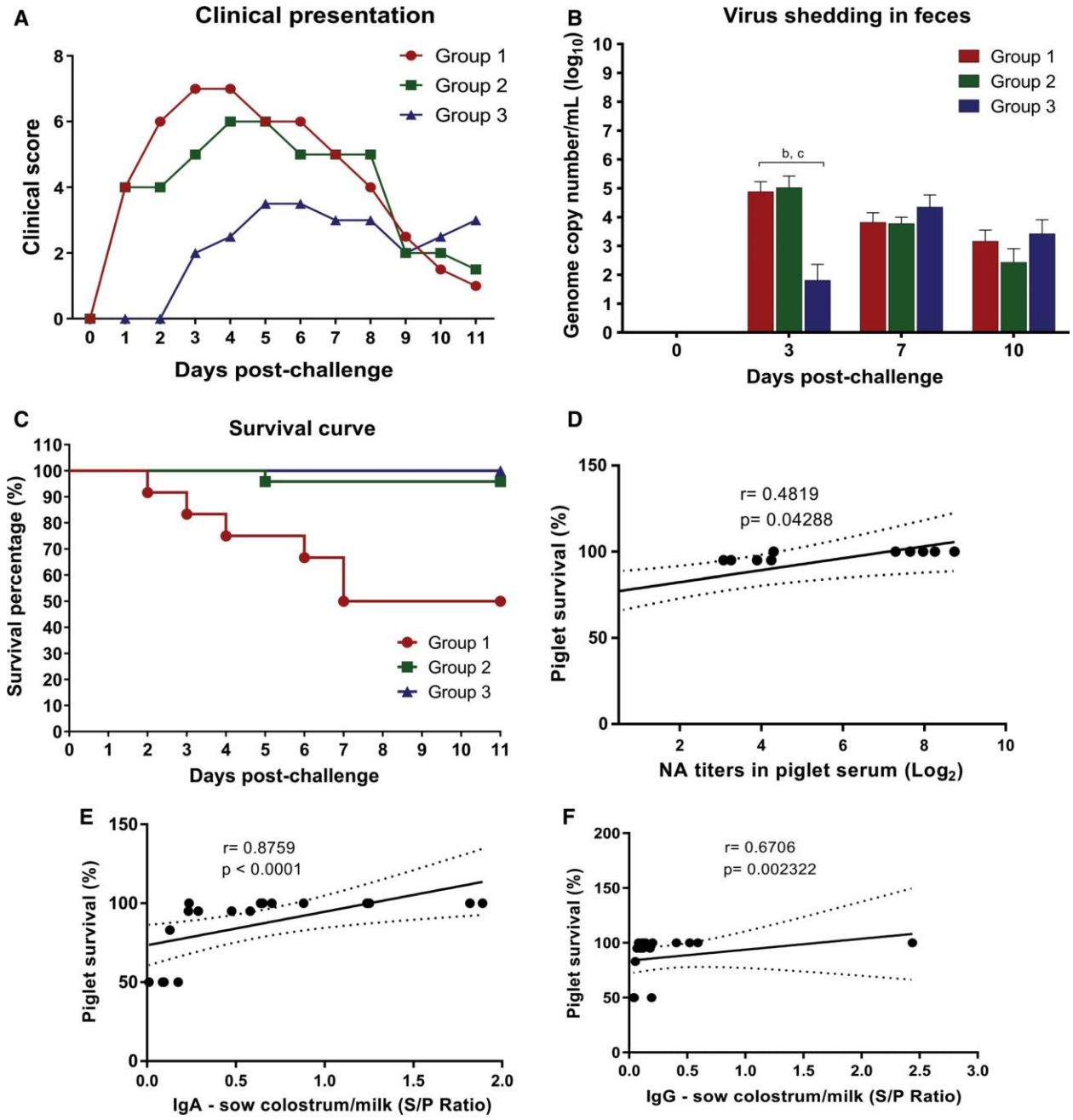

Figure 2.4 Clinical presentation, virus shedding, and piglet survival. (A) Daily average group clinical scores recorded after challenge infection with PEDV strain CO13. (B) Virus shedding as determined by PEDV RT-qPCR in piglet feces expressed as log10 genome copy number per milliliter. Data are presented as group means. Error bars represent +/- SEM. Statistical significance was determined using two-way ANOVA, and multiple comparisons were ran using Tukey's test. a, b, and c represent statistical significance for G1 vs. G2, G1 vs. G3, and G2 vs. G3, respectively. The significance level is < 0.01. (C) Survival curve demonstrating piglet mortality. The survival curve was generated using the Kaplan-Meier method. Statistical comparison between groups was performed using the log-rank test in GraphPad Prism 7. The P-value was adjusted using the Bonferroni method. (D) Correlation of piglet mortality with piglet group mean NA levels. Correlation of piglet mortality with group mean IgA antibodies in sow

colostrum/milk. (F) Correlation of piglet mortality with group mean IgG antibodies in sow colostrum/milk. Correlations were calculated using the Spearman method with 95% confidence interval using GraphPad Prism 7.

Discussion

In the present study, we assessed the ability of ORFV-PEDV-S to induce passive immunity against PEDV following immunization of pregnant gilts. One of the goals of the study was to assess whether immunization with ORFV-PEDV-S alone would be sufficient or if live exposure to PEDV would be required for protection of piglets born to immunized gilts. This underlies our experimental design, in which pregnant gilts were either immunized with ORFV-PEDV-S (G2) or with ORFV-PEDV-S followed by oral exposure to live PEDV (G3). Similar to our findings in 3-week-old weaned pigs [11], IM immunization of gilts with ORFV-PEDV-S elicited PEDV-specific IgG, IgA and NAs. As expected, the booster provided by live PEDV exposure in gilts from G3 led to higher antibody responses in serum, colostrum and milk of these animals. Notably, passive transfer of antibodies from gilts to piglets was observed in both G2 and G3, as PEDV-specific IgG, IgA and NAs were detected in serum of piglets born to immunized gilts following ingestion of colostrum and milk. NA antibodies detected in piglet serum showed a strong correlation with IgG and IgA levels (Fig. 2.3E and F). Although the antibody responses elicited by immunization with ORFV-PEDV-S alone were lower in comparison with those induced by immunization with ORFV-PEDV-S followed by live PEDV exposure, the levels of antibodies induced by IM immunization with ORFV-PEDV-S were sufficient to reduce neonatal mortality after oral challenge with PEDV.

Antibodies present in sow colostrum and milk are derived either from serum or produced locally in the mammary tissue [14], and transfer of pathogen-specific antibodies from the sow to the piglet via colostrum and milk is critical for protection against the pathogens during the

neonatal phase of the piglet's life. In this context, IgA plays a key role in protection against enteric pathogens such as PEDV, mainly because it is stable to proteolytic degradation in the intestine. By remaining in the gut lumen, pathogen-specific IgA can effectively inhibit/decrease virus infection/replication in the gut epithelium [8, 15,16,17]. The results of this study show that increasing levels of IgA in milk of immunized animals paralleled lower disease morbidity and mortality in piglets born to immunized gilts after challenge with PEDV. While severe PED and high mortality (50%) were observed in piglets born to control gilts in G1 (no antibody responses detected), piglets born to gilts in G2 and G3 (high levels of IgA in G2 and even higher in G3) showed less-severe clinical signs and reduced mortality rates (5 and 0%, respectively). These results suggest that PEDV-specific IgA detected in piglets in G2 and G3 may have contributed to reducing disease severity and mortality in piglets. The possibility that other antibody isotypes or T cell-mediated immunity may have played a role in protection, however, cannot be formally excluded.

One of the most important observations of our study was the reduced mortality in piglets from G2 (5% versus 50% in the control group, G1), which were born to gilts immunized IM with ORFV-PEDV-S. Notably, the lower piglet mortality in this group paralleled S-specific IgG, IgA and NA responses detected in the gilts and passively transferred to piglets. Together, these observations show that parenteral immunization with ORFV-PEDV-S is sufficient to induce protective levels of passive immunity against PEDV. These results corroborate the findings of previous studies in which PEDV specific-antibodies have been detected in serum and colostrum/milk and passively transferred to piglets after parenteral immunization with live, inactivated and/or subunit vaccine candidates [18]. Although the mechanism(s) underlying this phenomenon remain unknown, it is possible that systemic antibodies could be transferred from

the sow's serum to colostrum and milk and then to the piglets. Alternatively, the immunomodulatory properties of the ORFV vector used here could potentially lead to migration of antibody-secreting plasma cells to the mammary gland, resulting in local antibody production. Future studies assessing local immune cells in the mammary gland and antibody isotypes in the intestinal lumen of piglets born to immunized sows will be critical for dissecting the precise mechanism(s) underlying the protective immune responses elicited by IM immunization with ORFV-PEDV-S observed here.

In summary, this study demonstrates the ability of ORFV-PEDV-S to confer passive immunity against PEDV following immunization of pregnant gilts. Similar to our previous findings in 3-week-old weaned pigs [11], IM immunization of gilts with ORFV-PEDV-S elicited PEDV-specific IgG, IgA and NAs responses. Additionally, passive transfer of antibodies from gilts to piglets was observed, as PEDV-specific IgG, IgA and NAs were detected in serum of piglets born to immunized gilts following ingestion of colostrum and milk.

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CHAPTER 3

Protective efficacy of an orf virus-vector encoding the hemmaglutinnin and the nucleoprotein of influenza A virus in swine

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Abstract

Swine influenza is a highly contagious respiratory disease of pigs caused by influenza A viruses (IAV-S). IAV-S causes significant economic losses to the swine industry and because of its zoonotic potential it poses a constant threat to public health. Effective IAV-S vaccines are, therefore, needed and highly desirable. Here, we developed two recombinant orf viruses, one expressing the hemagglutinin (HA) gene (OV-HA) and the expressing the HA and the nucleoprotein (NP) genes of IAV-S (OV-HA-NP). The immunogenicity and protective efficacy of these two recombinant viruses were evaluated in pigs. Both OV-HA and OV-HA-NP recombinants elicited robust neutralizing antibody response in pigs. Additionally, both recombinant viruses elicited IAV-S-specific T-cell responses. However, the T-cell responses elicited by OV-HA-NP was higher than that elicited by OV-HA. Immunization with OV-HA induced Th2-biased immune response in pigs, whereas immunization with OV-HA-NP virus resulted in a Th1-biased immune response. Importantly, while pigs immunized with either OV-HA or OV-HA-NP were protected from IAV-S lung lesions, immunization with OV-HA-NP resulted in higher protective efficacy as evidenced by reduced virus shedding in nasal secretions and reduced viral load in the lung. This study demonstrates the potential of ORFV-based vectors for control of swine influenza virus in swine.

Introduction

Swine influenza is a highly contagious respiratory disease of pigs caused by influenza A viruses in swine (IAV-S). IAV-S is an enveloped, single stranded RNA virus of the family *Orthomyxoviridae*. The IAV-S genome consists of eight single-stranded negative-sense RNA segments encoding four structural (HA, NA, NP and M) and four non-structural (PB1, PB2, PA and NS) proteins. Influenza viruses are classified into subtypes based on the antigenicity of hemagglutinin (HA) and neuraminidase (NA) proteins present on the surface of the virus. There are thee recognized subtypes of IAV-S that are currently circulating in the US: H1N1, H1N2 and H3N2 (1). The H1N1 subtype is the major subtype that has been prevalent in the US swine population for decades; however, recent epidemiologic data suggests an increasing incidence of H1N2 and H3N2 subtypes (2, 3). IAV-S causes acute respiratory disease in pigs resulting in high morbidity (up to 100%). The mortality rate is usually low (1-4%) with most infected animals recovering within 3-7 days of infection (4, 5). The median yearly herd prevalence of IAV-S reported in the US is approximately 28%, but it can reach up to 57% in winter and spring months (6). IAV-S results in significant economic losses to the swine industry mainly due to weight losses, increased time to market, costs associated with treatment of secondary bacterial infections and mortality. This makes IAV-S one of the top three health challenges to the swine industry affecting pigs in all phases of production (7, 8). In addition to IAV-S pigs are also susceptible to infection with avian and human influenza A viruses thereby providing a niche for genetic reassortment between avian/human or swine influenza viruses. This poses a major threat for emergence of new subtypes as well as increases the risk of zoonotic transmission of IAVs. Therefore, effective prevention and control measures for IAV infections in swine have direct impacts on both animal and human health.

Currently, most available IAV-S vaccines are based on whole inactivated virus (WIV). However, these vaccines have not been able to effectively control IAV in swine and in some cases vaccine associated enhanced respiratory disease has been observed when there is an antigenic mismatch between vaccine strain and infecting strain (9). A live-attenuated influenza virus (LAIV) vaccine based on a virus containing a deletion of the NS1 gene, has been recently licensed for use in pigs in the US and may overcome some of the drawbacks of WIV vaccines (10). However, LAIV vaccines have the potential to reassort with the endemic viruses resulting in new influenza virus variants. Indeed, novel variants that arose from reassortment between the vaccine virus and endemic field strains have been recently reported (11). These observations highlight the need for safer and more efficacious IAV-S vaccine candidates. Here we investigated the potential of vectored vaccine candidates based on the parapoxvirus orf virus (ORFV) in controlling IAV-S in pigs.

Orf virus (ORFV) belongs to genus *Parapoxvirus* within the family *Poxviridae* (12) and is a ubiquitous virus that primarily causes a self-limiting mucocutaneous infection in sheep, goats and wild ruminants (13, 14). ORFV contains a double-stranded DNA genome with approximately 138 kbp in length and encodes 131 putative genes, including several with immunomodulatory (IMP) functions (15). Given ORFV IMP properties, the virus has long been used as a preventive and therapeutic agent in veterinary medicine (16, 17). Additionally, the potential of ORFV as a vaccine delivery platform against several viral diseases in permissive and non-permissive animal species has been explored by us and others (18–24). The use of ORFV recombinant vectors for vaccine delivery in swine, has also been previously explored and those formulations presented an excellent safety and efficacy profile. ORFV based vectored-vaccine candidates have been shown to induce protective immunity against pseudorabies virus (PRV),

classical swine fever virus (CSFV) and porcine epidemic diarrhea virus (PEDV) (22, 25–27). Among the features that make ORFV a promising viral vector for vaccine delivery in swine are : (i) its restricted host range, (ii) its ability to induce both humoral and cellular immune response (22, 28), (iii) its tropism which is restricted to skin keratinocytes with the absence of systemic infection, (iv) lack of vector-specific neutralizing antibodies which allows efficient prime-boost strategies using the same vector constructs (29, 30), and (v) its large genome size with the presence of many non-essential genes, which can be manipulated without severely impacting virus replication. Additionally, ORFV encodes several genes with well-characterized immunomodulatory properties. These include a homologue of interleukin 10 (IL-10) (31), a chemokine binding protein (CBP) (32), an inhibitor of granulocyte-monocyte colony stimulating factor GMC-CSF) (33), an interferon resistance gene (VIR) (34), a homologue of vascular endothelial growth factor (VGEF) (35), and inhibitors of nuclear-factor kappa-B (NF-ƙB) signaling pathway (36–39). The presence of these well-characterized immunomodulatory proteins allowed us to rationally engineer ORFV-based vectors with enhanced safety and immunogenicity profile for use in livestock species, including in swine (22–24).

Here we assessed the immunogenicity and protective efficacy of recombinant ORFV vectors expressing the HA protein alone or the HA and the nucleoprotein (NP) of IAV-S. While the HA protein contains immunodominant epitopes recognized by neutralizing antibodies (40, 41), the NP protein contains highly conserved immunodominant T-cell epitopes (42). We investigated whether co-expression of HA and NP would enhance the protective efficacy against IAV-S following intranasal challenge in pigs.

Material and methods

Cells and viruses. Primary ovine turbinate cells (OFTu), Madin-Darby canine kidney cells (MDCK) and swine turbinate cells (STU) were cultured at 37 °C with 5% CO_2 in minimum essential medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine and containing streptomycin (100 µg/mL), penicillin (100 U/mL and gentamycin (50 µg/ mL).

The ORFV strain IA82 (OV-IA82; kindly provided by Dr. Daniel Rock at University of Illinois Urbana-Champaign), was used as the parental virus to construct the recombinants and in all the experiments involving the use of wild-type ORFV. Wild-type and recombinant ORFV viruses were amplified in OFTu cells. Swine influenza virus H1N1 A/Swine/OH/24366/2007 (H1N1-OH7), kindly provided by Gourapura Lab was used for virus challenge, virus neutralization assay, hemagglutination inhibition (HI), and as a coating antigen for whole virus ELISA. The H1N1-OH7 virus was propagated in MDCK cells using DMEM containing TPCK-treated trypsin (2 µg/mL) and 25 mM HEPES buffer.

Generations of recombination plasmids. To insert the heterologous IAV-S gene in the ORFV121 locus, a recombination plasmid containing right and left flanking sequences of the ORFV121 gene were inserted into pUC57 plasmid. The HA gene of H1N1-OH7 virus was inserted between the ORFV121 flanking sequence in the pUC57 plasmid. The HA gene was cloned under the vaccinia virus (VACV) I1L promoter (5'-TATTTAAAAGTTGTTTGGTGAACTTAAATGG – 3') (43) and a flag-tag epitope (DYKDDDK) was fused to the amino terminus of the HA gene to detect its expression. The gene encoding green fluorescent protein (GFP) was inserted downstream of HA gene and used as a selection marker for recombinant virus purification. The GFP sequence was flanked by *loxp*

sequences 5'-ATAACTTCGTATAATGTATACTATACGAAGTTAT-3' to allow for removal of GFP by Cre recombinase following recombinant virus purification. This recombination cassette was named pUC57-121LR-SIV-HA-loxp-GFP (Fig 3.1A).

Similarly, another recombination cassette was generated to insert NP gene of IAV-S into the ORFV ORFV127 locus. A recombination cassette for ORFV127 was constructed as describe above with the ORFV127 left and right flanking regions being cloned into the pUC57-LoxP-GFP plasmid (pUC57-127LR-LoxP-GFP. The nucleoprotein (NP) gene of H1N1-OH7 was inserted between ORFV127 left and right flanks. The NP gene was cloned under the VACV vv7.5 promoter (44) and the HA epitope tag sequence (YPYDVPDYA) was fused at the amino terminus of the NP protein to detect its expression by the recombinant virus. This recombination cassette was named pUC57-127LR-SIV-NP-loxp-GFP (Fig 3.1B).

Generation of recombinant OV-HA and OV-HA-NP viruses. The HA gene of IAV-S was inserted into the ORFV121 locus of the ORFV genome by homologous recombination. Briefly, OFTu cells cultured in 6-well plate were infected with OV-IA82 with a multiplicity of infection (MOI) of 1. Three hours later, the infected cells were transfected with 2 µg of pUC57-121LR-SIV-HA-loxp-GFP using Lipofectamine 3000 according to the manufacturer's instruction (Invitrogen, catalog no: L3000-075). At 48 hours post- infection/transfection cell cultured were harvested, subjected to three freeze-and-thaw cycles. The ORFV recombinant expressing IAV-S HA was purified using plaque assay by selecting viral foci expressing GFP. After several rounds of plaque purification, the presence of HA gene and absence of ORFV121 gene was confirmed by PCR as described before (22, 24) and the insertion and integrity of the whole genome sequence of the recombinant was confirmed sequencing using Nextera XT DNA library preparation following by sequencing on the Illumina Mi-Seq sequencing platform. Once the

purified recombinant virus was obtained, the GFP selection gene was removed by using Cre recombinase treatment as described below. This recombinant is referred to as OV-HA throughout this manuscript.

Similarly, double gene expression vector containing the IAV-S HA and NP genes in ORFV121 and the ORFV127 gene loci (48), respectively was generated by homologous recombination. Both ORFV121 and ORFV127 are virulence determinants that contribute to ORFV IA-82 virulence in the natural host (39, 48). For this, infection/transfection was performed by infecting OFTu cells with the OV-HA recombinant virus and transfecting with pUC57-127LR-SIV-NP-loxp-GFP plasmid. The recombinant virus was purified using plaque assay as described above and following purification the GFP reporter gene was removed using the Cre recombinase treatment described below. The resulting recombinant ORFV vector expressing the HA and NP gene is referred to as OV-HA-NP in this manuscript.

The Cre/loxP recombination system was used to remove the GFP reporter gene from the OV-HA or OV-HA-NP recombinants. A plasmid pBS185 CMV-Cre , carrying the cre gene under the hCMV promoter was a kind gift from Brian Sauer (63) (Addgene catalog number : 11916) . OFTu cells were plated in a 24- well plate and 24h later transfected with 500 ng of the pBS185-CMV-Cre plasmid using Lipofectamine 3000 (Invitrogen, catalog num: L3000-075) according to the manufacturer's instructions. Approximately 24h after transfrections, cells were infected with ~ 1 MOI of the plaque purified recombinant viruses (OV-HA-GFP or OV-HA-NP-GFP). Approximately 48 h post-infection, the cre recombinase treated recombinant viruses were harvested and subjected to a second round of Cre treatment as described above. Following cre recombinase treatment, two to three rounds of plaque assays were performed to select foci lacking GFP expression and to obtain reporterless OV-HA or OV-HA-NP recombinant viruses.

Following markerless virus selection complete genome sequencing was performed to determine the integrity of ORFV and IAV-S sequences in the recombinant OV-HA and OV-HA-NP viruses.

Growth curves. Replication kinetics of OV-HA and OV-HA-NP recombinant viruses were assessed *in vitro* in OFTu and STU cells. Briefly, OFTu and STU cells cultured in 12-well plates were inoculated with OV-HA or OV-HA-NP with a multiplicity of infection (MOI) of 0.1 (multistep growth curve) or 10 (single-step growth curve) and harvested at 6, 12, 24, 48, 72 hours post-infection (hpi). Virus titers in cell lysates and supernatants were determined on each time point using Sperman and Karber's method and expressed as tissue culture infectious dose 50 ($TCID_{50}$) per milliliter (64).

Immunofluorescence. Immunofluorescence assay (IFA) was used to assess expression of the heterologous proteins by the OV-HA or the OV-HA-NP viruses. OFTu cells were inoculated with each recombinant virus (MOI of 1) and fixed with 3.7% formaldehyde at 48 hours pi. Then, cells were permeabilized with 0.2% PBS-Triton X-100 for 10 min at room temperature. Another set of samples which were not permealized were also tested side-by-side to compare the expression pattern between permeabilized and non-permeabilied cells. Flag-tag specific mouse antibody (Genscript, catalog no: A100187) and HA-tag specific rabbit antibody (Cell Signaling, catalog no: 3724S) were used as primary antibody to detect HA and NP protein respectively. Then, cells were incubated with Alexa fluor 594 goat anti-mouse IgG (H+L) secondary antibody (Invitrogen, catalog no: A11005) or Alexa fluor 488 goat anti-rabbit IgG antibody and cells were observed under fluorescence microscope.

Animal immunization and challenge studies. The immunogenicity of the two recombinant viruses (OV-HA and OV-HA-NP) was evaluated in 3-week old high-health pigs. A summary of

experimental design is presented in Table 3.1. Twenty-four pigs, seronegative for IAV-S, were randomly allocated into three experimental groups as follows: Group 1, sham immunized (n=8); Group 2, OV-HA immunized (n=8); Group 3, OV-HA-NP immunized (n=8). Immunization was performed by intramuscular injection of 2 ml of a virus suspension containing 10^7 $TCID_{50}$/mL in MEM. All animals were immunized on day 0 and received a booster immunization on day 21 post-immunization. All animals were challenged intranasally on day 35 post-immunization with 5 mL virus inoculum containing 6 X 10^6 $TCID_{50}$ of H1N1 A/Swine/OH/24366/2007 (H1N1-OH7) per animal. Animals were monitored daily for clinical signs of IAV-S. Serum and PBMC samples were collected on days 0, 7, 14, 21, 28-, 35-, 38- and 42-days post-immunization. Nasal swabs were collected on days 0, 1, 3, 7 post-challenge. The experiment was terminated on day 42 post-immunization or 7 days post-challenge. Whole lungs as a unit were collected from euthanized animals during necropsy and examined grossly for pathologic changes by a pathologist blinded to study groups. Animal immunization challenge studies were conducted at South Dakota State University (SDSU) Animal Resource Wing (ARW), following the guidelines and protocols approved by the SDSU Institutional Animal Care and Use Committee (IACUC approval no. 17-018A)

Group	Immunization	Number of animals	Immunization Days	Immunization Route	Challenge	Challenge Dose (Route)
1	Control	8	0, 21	IM	H1N1-OH7	6×10^6 $TCID_{50}$ (Intranasal)
2	OV-HA	8	0, 21	IM	H1N1-OH7	6×10^6 $TCID_{50}$ (Intranasal)
3	OV-HA-NP	8	0, 21	IM	H1N1-OH7	6×10^6 $TCID_{50}$ (Intranasal)

Table 3.1. Experimental design for immunization-challenge study

Virus neutralization (VN) assay. For VN assay, serum samples were heat inactivated for 30 minutes at 56 °C. Two-fold serial dilutions of serum were incubated with 200 $TCID_{50}$ of IAV-S H1N1-OH7 at 37 °C for 1 hour. This virus-serum complex was then transferred to a 96-well plate pre-seeded with MDCK cells 24 h earlier. After 1 hour of adsorption, virus-serum complex was removed and fresh DMEM containing 2 µg/mL of TPCK-treated trypsin was added to the cells. After 48-hour incubation at 37 °C, cells were fixed with 80% acetone. Virus positive MDCK cells were detected by immunofluorescence assay using a mouse monoclonal antibody targeting nucleoprotein (NP) of influenza virus (IAV-NP HB-65 mAb; kindly provided by Drs. Eric Nelson and Steve Lawson at SDSU). The virus neutralization titer was defined as the reciprocal of the highest dilution of serum where there was complete inhibition of infection/replication as evidenced by absence of fluorescent foci. Appropriate positive and negative control samples were included in all the plates.

Hemagglutination inhibition (HI) assay. For the hemagglutination inhibition assay, serial 2-fold dilution (starting dilution 1:4) were prepared in PBS. Then 4 HA units of H1N1-OH7 virus was added to the serum dilutions and incubated at room temperature for 1 hour. A solution (in PBS) of turkey red blood cells (containing 0.5% RBC) were added to the wells and allowed to

settle. The HI titer was calculated as the reciprocal of the highest dilution of sera that inhibited hemagglutination of turkey RBC.

Real-time reverse transcriptase PCR (rRT-PCR). Virus shedding in nasasl secretions and viral load in lungs was evaluated by rRT-PCR. Lung tissues were homogenized using tissue homogenizer by adding 10 mL of DMEM in 1 g of lung tissue. Viral nucleic acid was extracted from the nasal swabs and lung tissue homogenates using the MagMax Viral RNA/DNA isolation Kit (Life Technologies). The rRT-PCR tests were performed at Animal Disease Research and Diagnostic Lab (ADRDL), SDSU, SD. Genome copy numbers per milliliter were determined based on the relative standard curve derived from four-parameter logistic regression analysis (*R-square=0.9928, Root mean square error (RMSE)=1.0012*).

Virus isolation. Virus isolation was performed on the nasal swabs collected on day 0, 1, 3, and 7 post-challenge. Nasal swabs were filtered through a 0.22-micron filter and mixed with DMEM containing 2 µg/mL of TPCK-treated trypsin in 1:1 ratio. Then, 250 µL of this inoculum was added to 24-well plate containing MDCK cells. The cells were incubated for 1 hour at 37 °C. After 1 hour adsorption, 250 µL of DMEM was added to the wells and plate was incubated for 48 hours. After 48 hours, cell lysate was harvested, and two more blind serial passages were performed. After the third passage, the supernatant was collected, and the cells were fixed with 80% acetone. Immunofluorescence assay (IFA) was performed using IAV-NP mAb (IAV-NP HB-65) as primary antibody and Alexa fluor 594 goat anti-mouse antibody as secondary antibody (Invitrogen, catalog no: A11005). SIV infected cells were identified based on the presence of fluorescent foci.

ELISA. IAV-S-specific IgG, IgG1a and IgG2a immune response elicited by immunization with OV-HA or OV-HA-NP were assessed by whole virus ELISA. The antigen for coating the ELISA

plates was prepared by ultracentrifugation of SIV H1N1-OH7 using 30% sucrose gradient at 18,000 g for 1.5 hours. The virus pellet was resuspended in DMEM and UV inactivation of the virus was carried out using CL1000 UV crosslinker. Determination of the optimal coating antigen concentration and dilution of secondary antibodies were carried out by checkerboard titration.

To detect IAV-S specific total IgG, Immulon 1B ELISA plates (ThermoFisher Scientific, catalog no: 3355) were coated with 250 ng/well of concentrated and UV inactivated IAV-S virus and incubated at 37 °C for 2 hours. Then plates were washed three times with PBST (1X PBS with 0.5% Tween-20) and blocked with 200 µL/well of blocking solution (5% milk in PBST) and incubated overnight at 4 °C. Then, the plates were washed three times with PBST. Serum samples diluted in blocking solution at the dilution of 1:100 was added, and the plates were incubated for 1 hr at room temperature (RT). After, three washes with PBST, 100 µL of biotinylated anti-pig IgG antibody (Bethyl, catalog no: A100-104) diluted in blocking buffer (1:4000) was added to the plate and incubated for 1 hr at RT. Following three washes, HRP-conjugated streptavidin (Thermo Scientific , catalog no: 21136) diluted in blocking solution (1:4000) was added to plates and incubated for 1 hr at RT. Plates were washed again for three times with PBST and 100 µL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the plates (KPL, catalog no: 5120-0047). Finally, the colorimetric reaction was stopped by adding 100 µL 1N HCl solution per well. Optical density (OD) values were measured at 450 nm using a microplate reader. Cut-off value was determined as mean OD of negative serum samples plus three times of standard deviation (mean + 3SD).

For isotype ELISA, mouse anti-pig IgG1 (Biorad, catalog no: MCA635GA) and mouse anti-pig IgG2 antibody (Biorad, catalog no: MCA636GA) were used as secondary antibodies

and plates were incubated with biotinylated anti-mouse antibody (KPL, catalog no: 5260-0048) before incubating with streptavidin-HRP antibody. Endpoint titer ELISA using serial two-fold serial dilutions of serum samples were performed to determine endpoint titer of SIV-specific IgG1 and IgG2 antibody levels in the serum samples. Other procedures were similar to the total IgG ELISA as described above.

Flow-cytometry. IAV-S-specific T-cell response elicited by ORFV recombinants was evaluated by an intracellular cytokine staining (ICS) assay for interferon gamma (IFN-ɣ) and T-cell proliferation assay. For IFN-γ expression assay, cryopreserved PBMCs collected on day 35 post-immunization (0 dpc) were thawed and seeded at a density of 5 X 10^5 cells/well in 96-well plate. Cells were stimulated with UV inactivated IAV-S at MOI of 1. Additionally, cells were stimulated with concanavalin (ConA: 2 μg/ml) (Sigma, catalog no: C0412) plus phytohemagglutinin (PHA: 5 μg/ml) (Sigma, catalog no: 61764) as positive control and cRPMI (RPMI with 10% FBS) was added to the negative control wells. Protein transport inhibitor, Brefeldin A (BD Biosciences, catalog no: 555029), was added 6 hours after stimulation and the cells were incubated for 12 hours prior to flow cytometric analysis. For the proliferation assay, PBMCs (35 dpi) were stained with 2.5 μM carboxyfluorescin succinimidyl ester (CFSE; in PBS) (BD Horizon, catalog no: 565082). CFSE stained cells were seeded at a density of 5 X 10^5 cells/well in 96-well plate. The cells were stimulated as described above. After stimulation, the cells were incubated for 4 days at 37°C with 5% CO_2 prior to staining. Antibodies used for staining were : CD3+ (Mouse anti-pig CD3Ɛ Alexa Fluor 647; BD Pharmingen, catalog no: 561476), CD4+ (Mouse anti-pig CD4, Monoclonal Antibody Center (WSU), catalog no: 74-12-4), CD8+ (Mouse anti-pig CD8, Monoclonal Antibody Center (WSU), catalog no: 76-2-11), IFN-γ (Anti-pig IFN- γ, BD Biosciences, catalog no: 8025920). The stained cells were analyzed

using Attune NxT flow-cytometer. Results were corrected for background proliferation by subtracting mock-stimulated proliferation from the frequency of cells that responded under inactivated SIV stimulation. The percentage of responding cells was calculated as the percentage of total T cells (live CD3+ cells).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The normality of the data was tested using Shapiro-Wilk test. Comparison of means between the groups was done using two-way ANOVA for normal data or Kruskal Wallis test for non-normal data. Pairwise comparison was done using Tukey multiple comparison test. P value of less than 0.05 was considered significant. Flow cytometry data was analyzed using Flow Jo software.

Results

Construction of ORFV recombinants. The OV-HA recombinant virus was obtained by inserting the full-length HA gene of IAV-S into ORFV121 locus by homologous recombination between a transfer plasmid pUC57-121LR-SIV-HA-loxp-GFP and the parental ORFV strain IA82 (Fig 3.1A). The OV-HA-NP recombinant virus was obtained by inserting the full-length HA gene into ORFV121 locus and the NP gene into ORFV127 locus. The wild type ORFV strain IA82 was used to generate the OV-HA virus which served as a parental virus for the generation of the OV-HA-NP recombinant (Fig 3.1B). Expression of HA was driven by the vaccina virus (VACV) I1L promoter (43) and expression of the NP gene was driven by the VACV vv7.5 promoter (44). After infection with the parental virus and transfection with the transfer plasmid, the recombinant viruses were obtained and selected. Several rounds of plaque assays were performed to obtain purified recombinant viruses. Once the recombinant virus was purified and verified by PCR, the marker gene encoding for the green fluorescent protein (GFP)

was removed by using the Cre recombinase system. Whole-genome sequencing of these recombinant viruses was performed after Cre recombinase treatment. Sequencing results confirmed the integrity and identity of ORFV sequences and demonstrated the presence of HA gene and deletion of ORFV121 in OV-HA construct and the presence of HA and NP genes and deletion of ORFV121 and ORFV127 gene in OV-HA-NP construct.

Replication kinetics of OV-HA and OV-HA-NP viruses *in vitro*. Replication properties of both recombinant viruses (OV-HA and OV-HA-NP) were assessed *in vitro* in ovine fetal turbinate cells (OFTu) and swine turbinate cells (STU) cells using one-step and multi-step growth curves (Fig 3.1C). Cells were infected with an MOI of 0.1 or 10 and cell lysates were harvested at 6, 12, 24, 48, 72 hours post-infection. Both recombinants replicated very efficiently in natural host OFTu cells. However, replication of these recombinants was markdely impaired in the STU cells, a primary cell from the target swine species (Fig 3.1C). These results demonstrate that OV-HA and OV-HA-NP recombinant viruses do not replicate efficiently in swine cells, which increases the safety profile of the vector for use in pigs.

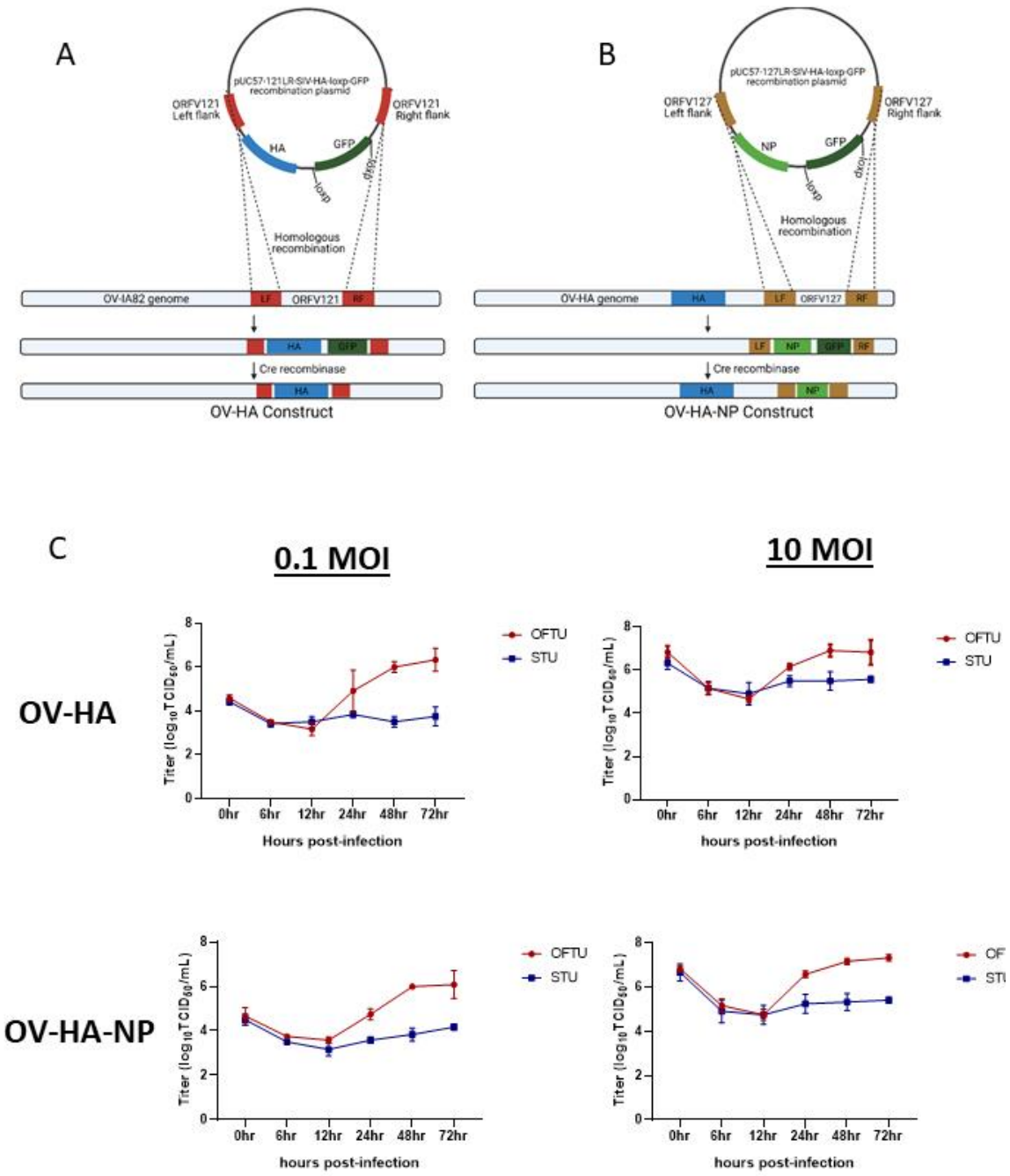

Figure 3.1. **Construction of ORFV recombinants and their replication kinetics**. **(A)** Schematic representation of homologous recombination between pUC57-121LR-SIV-HA-loxp-GFP plasmid and ORFV-IA82 genome. The recombinant virus was treated with Cre recombinase to remove GFP marker gene to obtain markerless OV-HA construct. **(B)** Schematic representation of homologous recombination between pUC57-127LR-SIV-NP-loxp-GFP plasmid and OV-HA genome. The recombinant virus was treated with Cre recombinase to obtain markerless OV-HA-NP construct. **(C)** Multi-step (0.1 MOI) and single step (10 MOI) growth curve of OV-HA and OV-HA-NP. OFTu or STU cells were infected with OV-HA and-HA-NP

recombinants and virus titers were calculated at 0, 6, 12, 24, 48 and 72 hours post-infection. Error bars represent SEM calculated based on three independent experiments.

Expression of heterologous proteins by OV-HA and OV-HA-NP recombinant viruses.

Expression of the HA protein by OV-HA recombinant and expression of the HA and NP proteins by OV-HA-NP virus was confirmed by immunofluorescence assay (IFA) and flow-cytometry. As shown in the figure 3.2A, OV-HA recombinant expressed high levels of HA and OV-HA-NP recombinant expressed high levels of HA and NP proteins (Fig 3.2A). Expression of HA and NP were also confirmed by flow cytometry (Fig 3.2C). The IFA was also performed in non-permeabilized cells. Both HA and NP proteins were detected in non-permeabilized cells; however, the levels were slightly lower than in permeabilized cells (Fig 3.2B). As expected, this decrease was more evident for NP protein than for the HA protein. These findings suggest that while a great proportion of the HA protein expressed by both OV-HA and OV-HA-NP recombinant viruses localizes to the cell surface, expression of the NP protein is mostly restricted to the intracellular compartment.

Immunogenicity of OV-HA and OV-HA-NP in pigs. To assess the immunogenicity of OV-HA and OV-HA-NP, pigs were immunized intramuscularly with two doses of OV-HA and OV-HA-NP at a 21-day interval (Fig 3.2A; Table 3.1). Antibody response were evaluated using a virus neutralization (VN) assay and a hemagglutination inhibition (HI) assay. One week after the first immunization, neutralizing antibodies were detected in both vaccinated groups, however the levels were significantly higher in OV-HA-NP vaccinated animals (Fig 3.2B). An anamnestic increase in neutralizing antibody titers was seen in both vaccinated groups one week after the

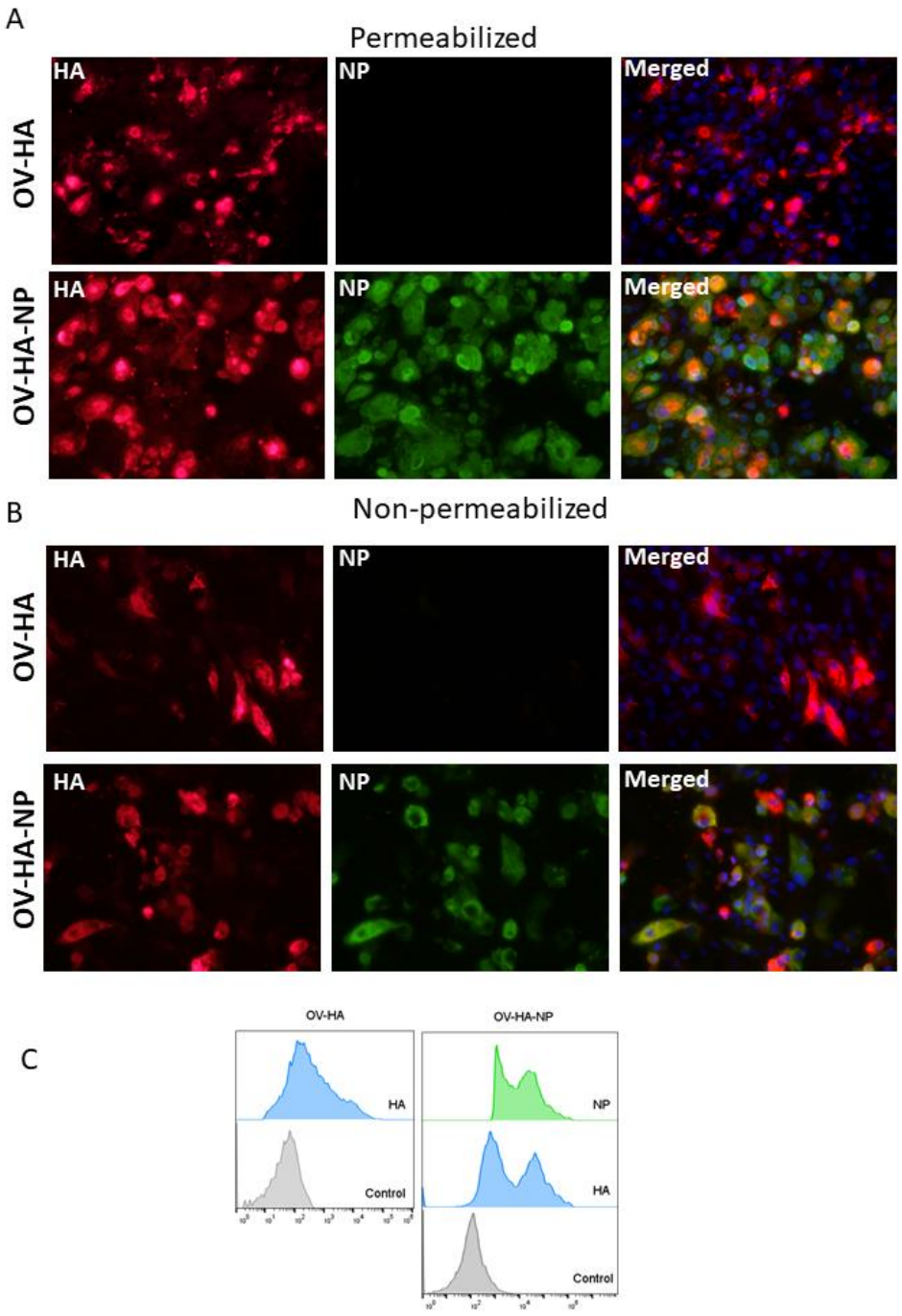

Figure 3.2. Expression of heterologous proteins by ORFV recombinants. (A) Immunofluorescence assay in permeabilized OFTu cells. Upper panel shows expression of HA protein and absence of NP protein in OV-HA recombinant. Lower panel shows expression of HA and NP protein by OV-HA-NP recombinant. **(B)** Immunofluorescence assay performed in non-permeabilized OFTu cells. Upper panel shows expression of HA by OV-HA recombinant and lower panel shows expression of HA and NP by OV-HA-NP recombinant. Blue fluorescence in merged images in panel A and B indicates nuclear staining by DAPI. **(C)** Expression of heterologous proteins by ORFV recombinants assessed by flow-cytometry. OFTu cells were infected with OV-HA, OV-HA-NP or Wild-type OV-IA82 as negative control. Infected cells were collected 48 hours post-infection, fixed and then stained with appropriate antibodies for flow cytometric analysis.

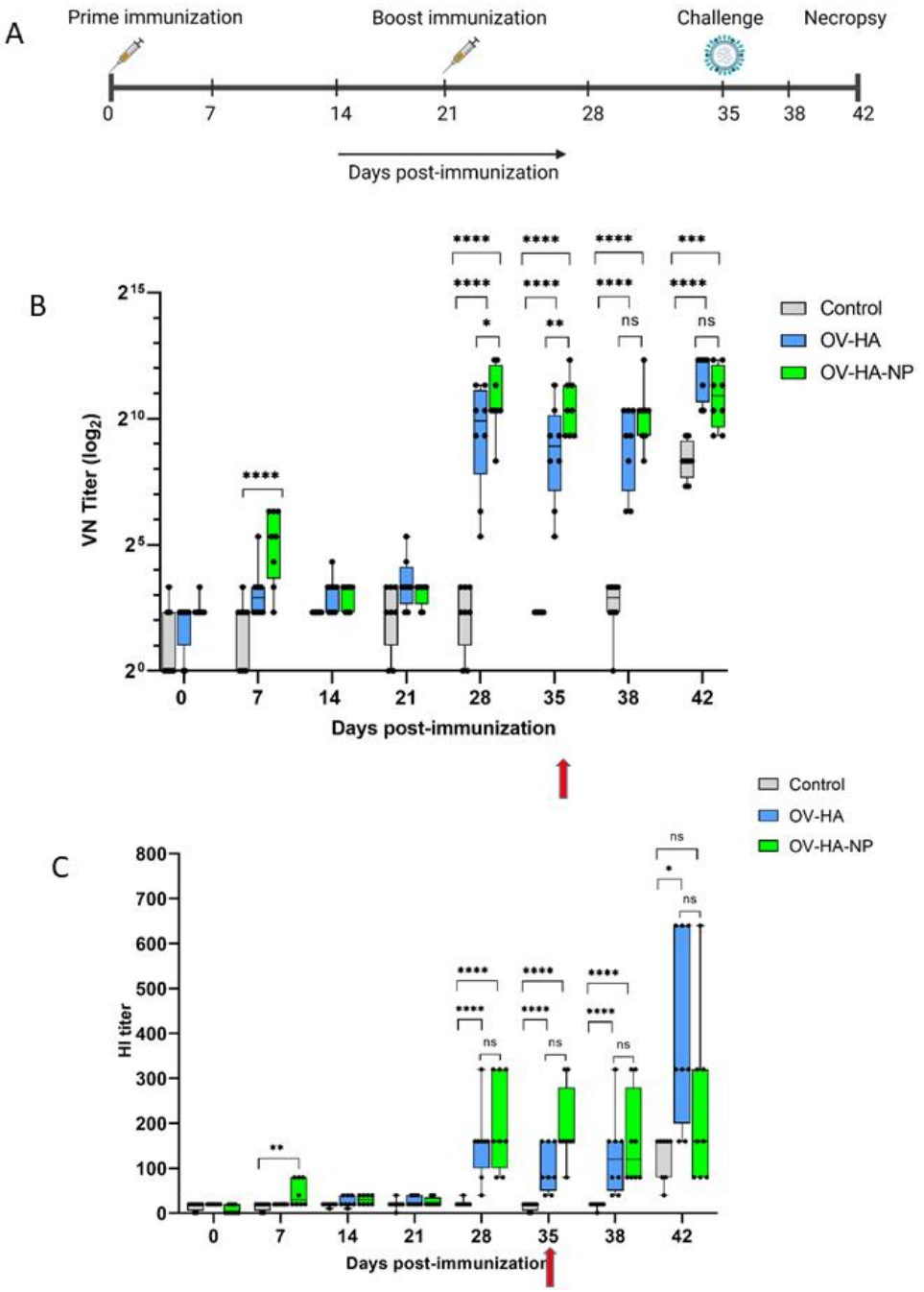

Figure 3.3. Immunization-challenge experiment design and humoral response to immunization. (A) A timeline of immunization-challenge experiment. **(B)** IAV-S specific neutralizing antibody response elicited by immunization with OV-HA and OV-HA-NP. **(C)** IAV-S specific humoral immune response induced by OV-HA and OV-HA-NP assessed by hemagglutination inhibition (HI) assay. Red arrow heads represent the day of challenge. The error bars represent SEM. VN titer shown in logarithmic scale for effective visualization. HI titer shown in liner scale. P-values: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

boost immunization (28 days post-immunization). The immunized animals maintained high level of neutralizing antibody levels until the end of the experiment (42 dpi, Fig. 3.3B).

Serological responses were also measured using an hemagglutination inhibition (HI) assay. The presence of HI antibodies were detected in OV-HA-NP group on day 7 pi. Similar to the VN results, an anamnestic increase in HI antibody titers was observed one week after the booster immunization in both groups (Fig 3.3C). Interestingly, the HI titers in the OV-HA group increased significantly after challenge, which is more evident a week after challenge (42 dpi). Such anamnestic increase in HI titers was not seen in OV-HA-NP-immunized animals, suggesting enhanced protection from IAV-S challenge (Fig 3.3C). Overall, these results demonstrate that immunization with OV-HA and OV-HA-NP viruses elicited high IAV-S specific neutralizing and HI antibody responses in immunized pigs.

IAV-S-specific IgG isotype responses elicited by immunization with OV-HA and OV-HA-NP viruses. IAV-S specific IgG responses were measured using a whole virus ELISA. Low levels of IAV-S-specific total IgG antibodies were detected in OV-HA and OV-HA-NP immunized groups on 21 days pi (Fig 3.4A). Similar to VN and HI assay, significantly higher levels of IgG antibodies was observed a week following the boost immunization (day 28 pi). Thereafter consistently higher levels of IgG were detected in serum of both OV-HA and OV-HA-NP immunized groups until the end of the experiment (Fig 3.4A). As expected, expression and delivery of the NP by the OV-HA-NP recombinant virus elicited higher levels of IgG antibodies in immunized pigs after the booster immunization on day 21 pi when compared to those observed in OV-HA-immunized animals ($P < 0.0001$, Fig 3.4A).

The endpoint titer of IgG1 and IgG2 isotype antibodies elicited by immunization with OV-HA and OV-HA-NP were determined by an isotype ELISA performed on serum samples collected.

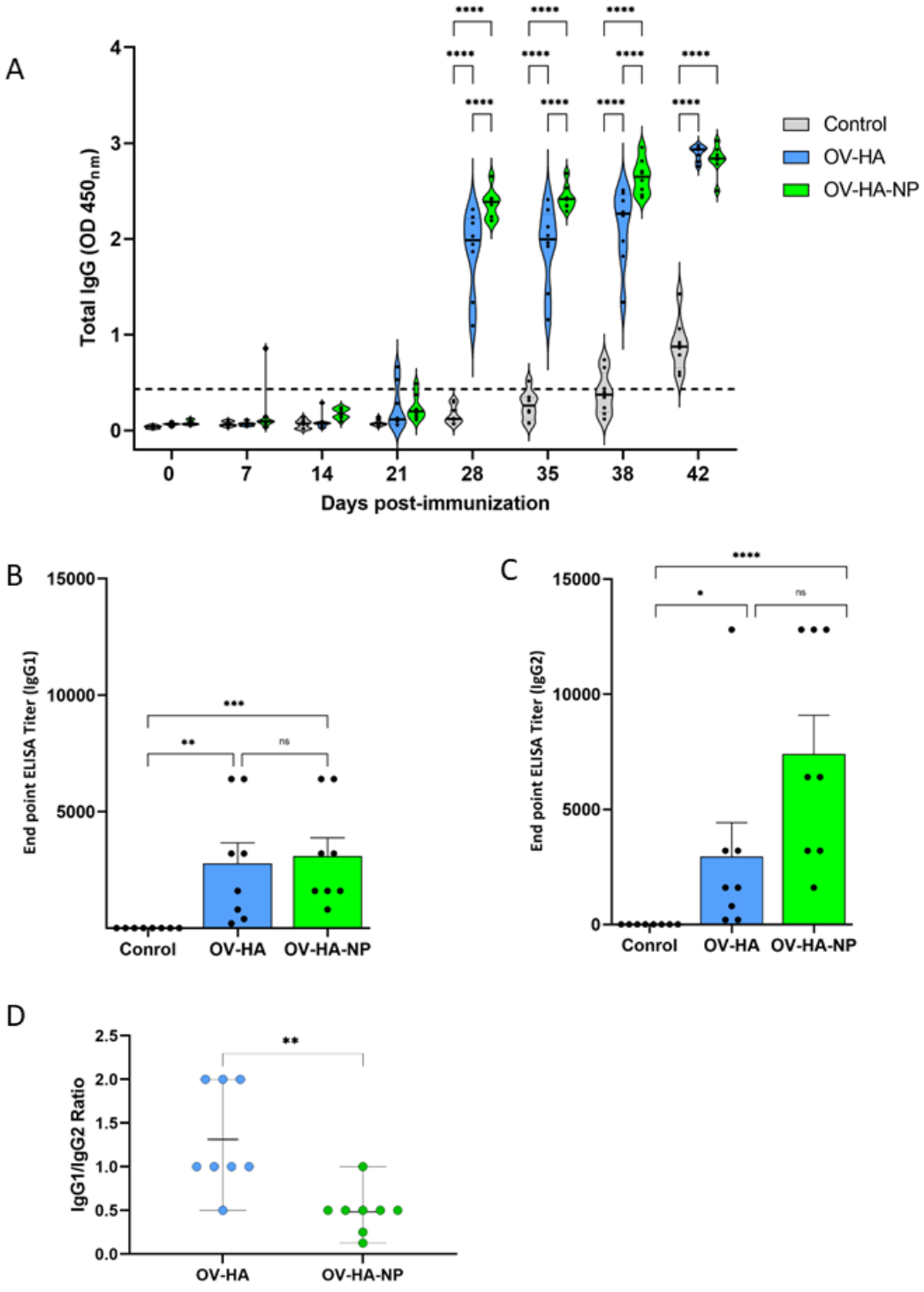

Figure 3.4. IAV-S specific IgG responses to immunization. (A) Total serum IgG level elicited by OV-HA and OV-HA-NP immunization assessed by ELISA. **(B)** Isotype ELISA demonstrating serum IgG1 endpoint titers elicited by immunization **(C)** Isotype ELISA demonstrating serum IgG2 endpoint titers elicited by immunization **(D)** IgG1/IgG2 ratio in immunized animals. The IgG1 and IgG2 titer was determined by endpoint ELISA. Each dot represents IgG1/IgG2 ratio of an individual animal. Middle bar represents mean ratio and upper and lower bars represent range. P-values: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

on 35 days pi. Immunization with OV-HA and OV-HA-NP viruses elicited similar levels of

IgG1 response, however, significantly higher titers of IgG2 antibodies were detected in OV-HA-NP-immunized animals when compared to IgG2 titers detected in OV-HA-immunized animals (Fig 3.4B and 3.4C). The IgG1/IgG2 ratio calculated based on the endpoint titers detected in each group was 1.31 (i.e. > 1) for the OV-HA group and 0. 48 (i.e. <1) for the OV-HA-NP group (Fig 3.4D). The IgG1/IgG2 ratio in OV-HA-NP group was significantly lower than in the OV-HA group (P = 0.0048, Mann-Whitney test). Together these results suggest that the immune response in OV-HA group is mostly Th1 biased. In contrast, immune response was Th2 biased on the OV-HA-NP group as indicated by higher levels of IgG2 antibodies in the serum of OV-HA-NP immunized animals.

Cellular immune responses elicited by immunization with OV-HA and OV-HA-NP. IAV-S-specific T-cell responses elicited by immunization with OV-HA and OV-HA-NP viruses was assessed on peripheral blood mononuclear cells (PBMCs) collected on 35 days pi (pre-challenge infection). The frequency of different T-cell subsets secreting IFN-γ following re-stimulation with IAV-S was measured using intracellular cytokine staining (ICS) assays. Upon singlet selection, live/dead cell discrimination, IFN-γ expression by different T-cell subsets including total T-cells (CD3+), CD4+ T-cells (CD3+/CD4+), CD8+ T-cells (CD3+/CD4+/CD8+), double positives (CD3+/CD4+/CD8+) and double negative T-cells (CD3+/CD4-/CD8-) was assessed (Figure 3.5). Animals immunized with either OV-HA or OV-HA-NP had significantly higher percentage of $CD3^+$ T-cells secreting IFN- γ when compared to the non-immunized control animals (Fig 3.5A). Notably, within the vaccinated animals, OV-HA-NP group presented a significantly higher frequency of IFN-γ secreting $CD3^+$ T-cells than the OV-HA group (*P=0.0055*). The animals in the OV-HA-NP group presented higher frequency of IFN-γ secreting CD3+/CD4+ T-cells, however, the differences between the groups was not statistically

significant. Both immunized

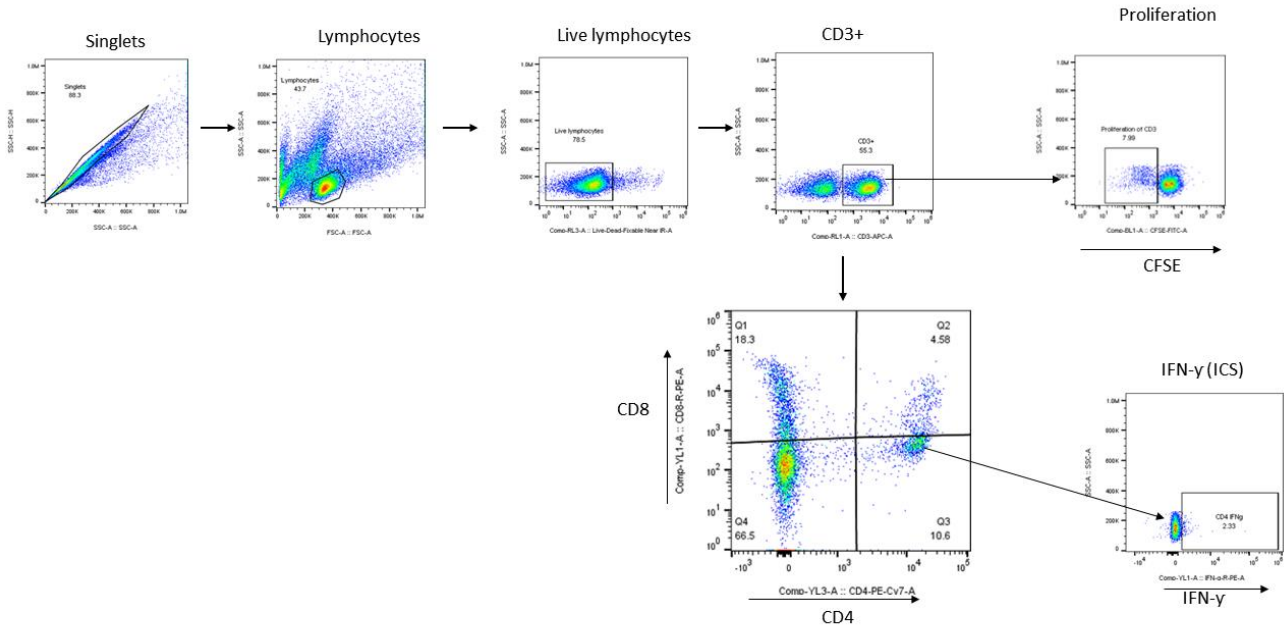

Fig 3.5 Gating strategy used in flow cytometry experiments. First, singlets were selected. Within the singlet population lymphocytes were gated. Live cells within the lymphocytes were selected. CD3 population within lymphocytes were gated. Then, within CD3 population different T-cells subpopulations were discriminated based of the CD4 and CD8 marker. Proliferation of all these T-cells subpopulations were determined based on dilution of CFSE and percentage of T-cell secreting IFN gamma was determined based on intracellular cytokine staining.

groups presented increased frequencies of CD3+/CD8+, CD3+/CD4+/CD8+ (double positives) and CD3+/CD4-/CD8- (double negative) IFN-γ secreting T-cell subsets when compared to the control sham-immunized group (Fig 3.6A).

IAV-S-specific T-cell responses were also evaluated by the carboxyfluorescein succinimidyl ester (CFSE) dilution assay to determine the specific T-cell subsets proliferating upon re-stimulation of PBMCs with inactivated IAV-S. As described above for the IFN-γ ICS, upon singlet selection and dead cell exclusion, proliferation by the major swine T-cell subsets was evaluated (Fig 3.6B). While proliferation of CD3+ T-cell subset was observed in animals immunized with OV-HA or OV-HA-NP, significant proliferation of CD3+ T-cells was observed in OV-HA-NP group upon recall stimulation (P=0.0095; Fig 3.6B). Additionally, a significant increase in the proliferation of CD3+/CD8+ T-cell subset was observed in the OV-HA-NP group (*P=0.0217*, Fig 3.6B). An increase in proliferation of CD3+/CD4+ T-cells was also observed (Fig 3.5B); however, the differences between the treatment groups were not statistically significant (Fig 3.6B). Overall, these results show that both OV-HA and OV-HA-NP group were able to induce IAV-S-specific T-cell responses in the immunized animals. As expected, T-cell responses elicited by immunization with the OV-HA-NP construct was higher than those observed in animals immunized with the single gene OV-HA construct.

Protective efficacy of OV-HA and OV-HA-NP viruses intranasal IAV-S challenge. The protective efficacy of OV-HA and OV-HA-NP were evaluated upon intranasal challenge with IAV-S (after day 35 pi). Virus shedding was assessed in nasal secretions and viral load and pathology were evaluated in the lung. Nasal swabs were collected on days 0, 1, 3, and 7 post-challenge (pc) and IAV-S RNA levels were investigated in nasal secretions using real-time

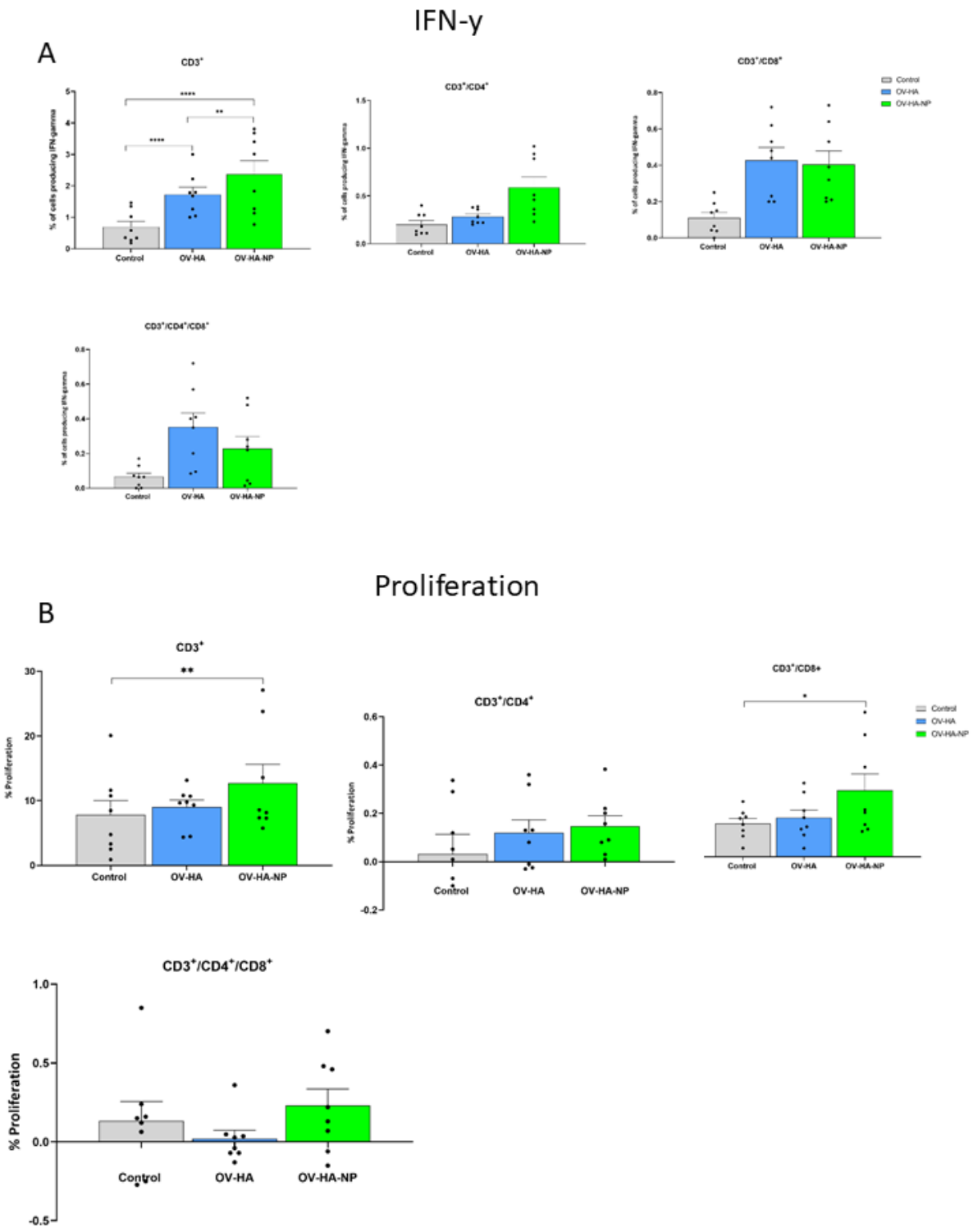

Figure 3.6. T-cell immune response to immunization. (A) IFN-γ production by different T-cell subsets on 35 dpi following recall stimulation with inactivated IAV-S. **(B)** T-cells proliferation following stimulation with inactivated IAV-S measured by CFSE dilution assay. Data represents group means and error bars represent SEM. P-values: **P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

reverse transcriptase PCR (rRT-PCR). On day 1 pc, significantly lower IAV-S genome copy numbers – indicating reduced virus shedding – was detected in both OV-HA and OV-HA-NP immunized groups when compared to the control sham immunized group (Fig 3.7A). Only two animals (2/8) in the OV-HA-NP group were positive for viral RNA on day 1 pc. On 3 dpc, while all animals in control group (8/8) were positive and presented high genome copy numers of IAV-S in nasal secretions, only three animals (3/8) in OV-HA-NP were positive for viral RNA (Fig 3.7A). Notably, the amount of IAV-S RNA shed by OV-HA-NP-immunized animals were significantly lower than the amount shed by control or OV-HA immunized animals. It is also important to note that animals in OV-HA group had significantly lower level of viral RNA than control group on day 3 pi (Fig. 3.7A). On day 7 post-challenge, all animals (8/8) in the control sham-immunized group were still shedding IAV-S in nasal secretions, while only two animals (2/8) in the OV-HA-immunized group were positive presenting low viral RNA copy numbers in nasal secretions. Notably, none of the animals in the OV-HA-NP-immunized group were shedding IAV-S in nasal secretions on day 7 pi (Fig 3.7A). These results demonstrate that immunization with OV-HA and OV-HA-NP resulted in decreased virus shedding and shorter duration of virus shedding in nasal secretions following intranasal IAV-S challenge. Notably, these differences were more pronounced in OV-HA-NP-immunized animals.

Shedding of infectious IAV-S was also assessed in nasal secretions collected on days 0, 1, 3, and 7 post-challenge. For this, virus isolation was performed in MDCK cells. Each sample was subjected to three blind passages in MDCK cells. An immunofluorescence assay using an IAV-S NP-specific monoclonal antibody was performed on the third passage to confirm isolation of IAV-S. On day 1 pc, 4 (50%) animals in the control group were positive for IAV-S, while none of the animals from the OV-HA and OV-HA-NP group were positive on VI (Table 3.2) .

On day 3 pc, 7 (87.5%) animals were positive in the sham-immunized control group; 3 (37.5%) animals were positive in OV-HA immunized group and 1 (12.5%) animal was positive in OV-HA-NP-immunized group. Statistical analysis confirmed that there was a significant difference in the number of IAV-S positive animals between control group and OV-HA-NP group on 3 dpc (P= 0.0101 Fisher's exact test) (Table 3.2). Virus was not isolated from any of the animals on day 7 post-challenge (Table 3.2) . These results indicate that both OV-HA and OV-HA-NP recombinants were able to reduce virus replication and shedding in the immunized animals. Importantly, detection of infectious virus in only one out of eight animals in OV-HA-NP groups highlingts the robust protection provided by immunization of pigs this recombinant virus.

Viral load was assessed in the lung of control and immunized pigs on day 7 dpc by using rRT-PCR. While high amounts of IAV-S RNA were detected in the lung of animals in the control sham-immunized group, immunization with OV-HA or OV-HA-NP led to a marked decrease in viral load in the lung (Fig 3.7B). Notably, only one animal (1/8) in OV-HA-NP group and two animals (2/8) in OV-HA group presented IAV-S RNA in lung, whereas all the animals in control group (8/8) were positive for IAV-S RNA. Significantly lower IAV-S RNA loads were detected in the lung of immunized animals when compared to control animals (Fig 3.7B)

In addition to viral loads pathological changes were also evaluated in the lung of all animals in the study. At necropsy, macroscopic lesions in the lung were characterized by a pathologist who was blinded to the experimental groups. A summary of the gross lung lesions is provided on Table 3.3. All animals in the control group presented characteristic plum-colored consolidated areas mostly on the cranioventral areas and interstitial pneumonia. Mild lobular consolidation and interstitial pneumonia was present in 2 animals in OV-HA group and 2

animals in OV-HA-NP group. As expected, the lesions were primarily observed in animals having relatively lower levels of neutralizing antibody titers (Table 3.3). No microscopic lesions were observed in any animals.

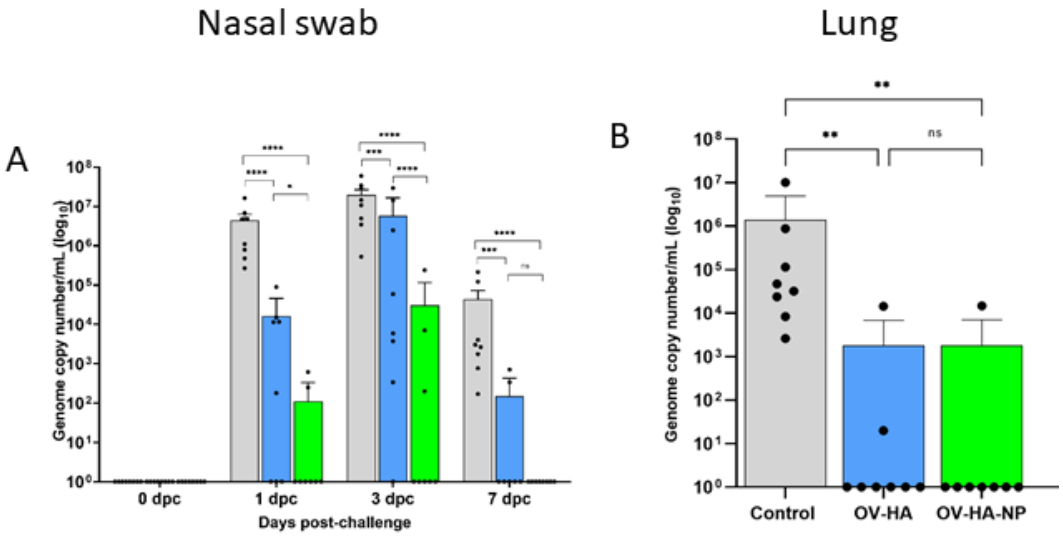

Figure 3.7. Protective efficacy of OV-HA and OV-HA-NP against IAV-S challenge. (A) IAV-S viral RNA shedding in the nasal swab determined by RT-qPCR and expressed as log10 genome copy number per milliliter. **(B)** IAV-S viral load in the lung determined by RT-qPCR and expressed as log10 genome copy number per milliliter. Data represents group mean and error bars represent SEM**.** P-values: **P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001*.

Groups	**0 dpc**	**1 dpc**	**3 dpc**	**7 dpc**
Control	0/8	4/8 (50%)	7/8 (87.5%)	0/8
OV-HA	0/8	0/8	3/8 (37.5%)	0/8
OV-HA-NP	0/8	0/8	1/8 (12.5%)	0/8
P-values	-	*[a]P =0.0769* *[b]P=0.0769*	*[a]P= 0.1189* *[b]P= 0.0101**	-

Table 3.2. Virus isolation from the nasal swabs
[a]P-value determined by Fisher's exact test between Control and OV-HA group
[b]P-value determined by Fisher's exact test between Control and OV-HA-NP group
*Statistically significant difference at *P < 0.05*

Control			OV-HA			OV-HA-NP		
Animal ID	**Gross Lesions**	**VN Titer[a]**	**Animal no**	**Gross Lesions**	**VN Titer[a]**	**Animal no**	**Gross Lesions**	**VN Titer[a]**
22	Lobular consolidation on the left cranioventral areas	<1:5	20	Mild lobular consolidations on both right and left lung	1:80	21	No lesions	1:5120
23	Lobular consolidation present on ventral areas	<1:5	24	No lesions	1:320	25	No lesions	1:2560
29	Lobular consolidation mostly present on cranioventral surface and interstitial inflammation on the left lobe	<1:5	26	Mild lobular consolidation on both sides of the lung	1:40	27	No lesions	1:640
30	Lobular consolidation mostly present on cranioventral area	<1:5	33	No lesions	1:640	28	No lesions	1:1280
31	Lobular consolidation mostly present on cranioventral area	<1:5	34	No lesions	1:2560	32	No lesions	1:1280
39	Lobular consolidation mostly present on cranioventral area	<1:5	35	No lesions	1:1280	37	Very mild lobular consolidation	1:640
40	Lobular consolidation mostly present on cranioventral area	<1:5	36	No lesions	1:640	38	Congestion on apical lobe with mild interstitial pneumonia	1:640
48	Lobular consolidation on both sides of	<1:5	49	No lesions	1:320	41	No lesions	1:2560

	the cranioventral area							

Table 3.3. Pathological and serological findings post-IAV-S-challenge in immunized pigs.

[a]VN titer measured on day 35 post-immunization.

All animals were terminated and examined on day 7 post-challenge and evaluated by a pathologist blinded to the study.

Presence of cross-reactive antibodies in OV-HA or OV-HA-NP immunized pigs

We further asked if the antibodies induced by OV-HA or OV-HA-NP were able to neutralize recent IAV-S (H1N1) isolates belonging to different clades. Ten different H1N1 strains isolated between 2017-2020 were used for cross-neutralization assay and these viruses represent different clades that are circulating globally (Table 3.4). Phylogenetic trees were constructed based on amino acid sequences of HA (Fig. 3.8A) and NP (Fig. 3.8B) proteins to evaluate genetic relatedness of these ten viruses with vaccine and challenge virus used in this study. The HA and NP sequence used to construct OV-HA or OV-HA-NP was based on A/Swine/OH/511445/2007 and this HA/NP sequence share 100% identity with HA/NP sequence of the challenge virus (A/Swine/OH/24366/2007) and cluster together in the phylogenetic trees (Fig. 3.8A and Fig 3.8B, Table 3.4). The amino acid difference between vaccine HA sequence and ten isolates used for cross-neutralization assay ranges from 3.1 % to 27 %, whereas the amino acid sequence of NP is more conserved and this difference ranges from 2.2 % to 3.4 % (Table 3.4).

To further profile the breath of neutralizing activity, serum samples collected on 35 days post-immunization was used for the cross-neutralization assay using the ten isolates described above. The serum from OV-HA-NP group was able to neutralize six out of ten isolates, whereas the serum from OV-HA immunized animals neutralized two isolates (Fig. 3.8C). These viruses that are neutralized by the serum from OV-HA-NP serum belong to gamma, gamma-2-beta-like, pdm and beta clades (Figure 3.8C; Table 3.4). The serum from OV-HA or OV-HA-NP group were able to neutralize H1N1 isolates that share more than 92% HA sequence similarity with the vaccine HA sequence. An exception to this is A/Swine/Oklahoma/A02245707/2020, which shares only 88.6% amino acid identity to the vaccine HA sequence but is still neutralized by the serum from both OV-HA and OV-HA-NP group. It is important to note that the cross-neutralizing antibody titers were low to moderate when compared to the titers against the challenge virus. Overall, these results show that antibody induced by OV-HA or OV-HA-NP have cross-neutralizing activities which can neutralize viruses that differ antigenically from the vaccine or challenge virus used in this study.

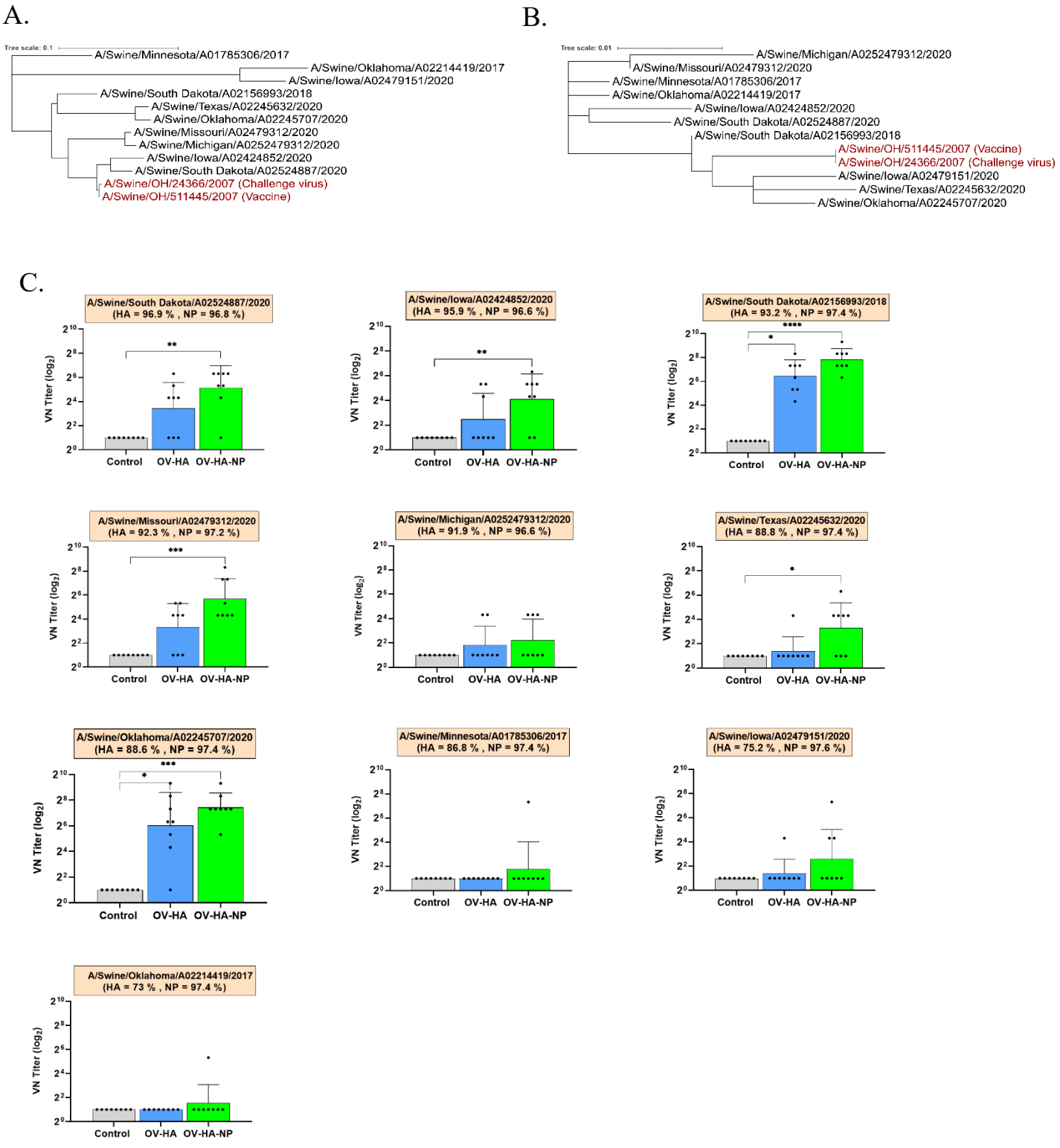

Figure 3.8. Cross-neutralization assay. (A) Phylogenetic tree based on the HA amino acid sequence **(B)** Phylogenetic tree based on NP amino acid sequence **(C)** Cross neutralizing antibody titers against different IAV-S isolates. The value in the parenthesis indicates amino acid similarity with vaccine HA/NP amino acid.

Virus	Year	Clade	HA similarity§	NP similarity¥
A/Swine/South Dakota/A02524887	2020	gamma	96.9	96.8
A/Swine/Iowa/A02524852	2020	gamma	95.9	96.6
A/Swine/South Dakota/A02156993	2018	gamma-2-beta-like	93.2	97.4
A/Swine/Missouri/A02479312	2020	pdm	92.3	972
A/Swine/Michigan/A02524810	2020	pdm	91.9	96.6
A/Swine/Texas/A02245632	2020	beta	88.8	97.4
A/Swine/Oklahoma/A02245707	2020	beta	88.6	97.8
A/Swine/Minnesota/A01785306	2017	alpha	86.8	97.4
A/Swine/Iowa/A02479151	2020	delta1a	75.2	97.6
A/Swine/Oklahoma/A02214419	2017	delta1b	73	97.4
A/Swine/OH/24366 (Challenge)	2007	gamma	100	98.6
A/Swine/OH/511445 (Vaccine)	2007	gamma	100	100

Table 3.4. Description of the isolates used in cross-neutralization assay.

§Percentage of HA amino acid similarity with the HA sequence used in OV-HA and OV-HA-NP
¥ Percentage of NP amino acid similarity with the NP sequence used in OV-HA-NP

Discussion

In this study we explored the potential of ORFV recombinants expressing the HA or HA and NP proteins of IAV-S in providing protection against intranasal challenge in swine. Previous work from our group have shown that rational vector design by deleting well-characterized immunomodulatory genes of ORFV is useful in developing highly effective vaccine delivery platforms resulting in safe and highly immunogenic vaccine candidates. One of the well characterized ORFV IMPs is ORFV121 , which encodes an NF-κB inhibitor that determines ORFV virulence and pathogenesis in the natural host (39). We have already developed highly immunogenic vaccine candidates for porcine epidemic diarrhea virus (PEDV) and rabies virus

(RabV) by inserting appropriate protective antigens (spike glycoprotein for PEDV; rabies glycoprotein for RabV) in the *ORFV121* gene locus. Given the immunogenicity and safety profile of the OV-PEDV-S and OV-RABV-G recombinant virus in swine, here we constructed an OV-HA recombinant by inserting the HA gene of IAV-S virus in ORFV121 locus. Moreover, to potentially enhance T-cell immune responses elicited by the vaccine we generated a second recombinant virus expressing the IAV-S NP protein in addition to the HA protein. For this, another well-characterized ORFV IMP, the ORFV127 was selected as an insertion site for the NP gene. ORFV127 encodes a viral IL-10 homolog (15, 45), which is known to have anti-inflammatory and immunosuppressive activities that may favor immune evasion of the orf virus (46, 47) and most importantly the protein encoded by ORFV127 is known to contribute to ORFV virulence in the natural host (48). Using this approach we tested the hypothesis that simultaneous deletion of two ORFV IMP genes ORFV121 and ORFV127 and concurrent insertion of two highly immunogenic protective antigens of IAV-S (HA and NP) should enhance the immunogenicity of the recombinant virus in swine and provide higher protective efficacy from IAV-S challenge. While the data presented here show that both recombinants OV-HA and OV-HA-NP induced robust immune response against IAV-S in pigs, the immunogenicity and protective efficacy of OV-HA-NP was indeed higher than that elicited by the OV-HA recombinant which substantiates our hypothesis.

Following challenge infection, we observed interesting differences in the antibody responses elicited by OV-HA and OV-HA-NP immunization. As expected, intranasal challenge with IAV-S in sham immunized pigs resulted in anamnestic VN responses. Notably, in the immunized groups the VN antibody titers increased by a greater magnitude in the OV-HA group than in the OV-HA-NP group. In the OV-HA group, the geometric mean VN titers were 380.5 and 3319.9

on days 0 and 7 pc respectively, indicating a 9-fold increase in the VN titer after challenge. Whereas in OV-HA-NP group, the geometric mean VN titers were 1395.8 and 1810.19 on days 0 and 7 pc respectively, indicating only a modest 1.2-fold increase in VN titers pc. Importantly, the VN titers in OV-HA-NP immunized group increased only in the two animals that had the lowest VN titer on day 0 pc. The VN titers in the remaining 6 animals in the OV-HA-NP group remained constant following challenge infection. These results demonstrate that immunization with the OV-HA-NP recombinant virus provided robust immune protection against intranasal IAV-S challenge, with most animals not seroconverting to the challenge virus.

The importance of T-cells in influenza virus clearance and their cross-reactive potential has been well documented (49, 50). In this context, CD4+ T-cells help with activation, differentiation and antibody production by virus-specific B cells (51). Additionally, CD4+ helper cells also play an important role in CD8+ cytotoxic T cell activation. Activated CD8+ cytotoxic T-cells fucntion in virus clearance by killing infected cells (52). The NP protein of influenza virus is known to contain several immunologically dominant T-cell epitopes and it is the main antigen recognized by cytotoxic T- lymphocytes (CTL) during influenza A virus infections (42, 53–56). The Immune Epitope Database and Analysis Resource, a manually curated database of experimentally characterized immune epitopes, has recorded 248 T-cells epitopes for nucleoprotein (NP) of influenza virus. Given that NP is relatively conserved among influenza viruses – including IAV-S, this protein has been one of the target viral antigens for the development of universal influenza vaccine candidates. Because of these important immunological properties, we have developed and evaluated the OV-HA-NP construct expressing both HA and NP proteins. We found that cell mediated immune responses were enhanced by co-delivery and expression of IAV-S HA and NP by OV-HA-NP in pigs when

compared to OV-HA group. A significantly higher frequency of CD3+ T-cells proliferated and expressed IFN-γ upon re-stimulation with IAV-S in the OV-HA-NP-immunized group. Importantly, immunization with OV-HA-NP resulted in a increased frequency of CD3+/CD8+ T cells upon restimulation with IAV-S. While overall T-cell responses were higher in OV-HA-NP group, an increase in T-cell response was also seen in OV-HA group when compared to the sham immunized group, as evidenced by increase in IFN-γ secreting CD3+ T-cell population following antigen stimulation. This can be explained by the fact that HA protein also contains several T-cells epitopes, the majority of which are CD4+ T-cell epitopes (57, 58).

Depending upon the type of antigenic stimulation, CD4+ helper T-cell precursors (Th_o) can either differentiate into Th1- or Th2- helper cells. Th1 cells secrete several cytokines including IFN-γ and IL-12 which help in cell mediated immunity, whereas Th2 cells secrete cytokines like IL-4, IL-6 which contribute to antibody mediated immunity (40, 59). Importantly, IgG isotype expression is also controlled by the different cytokines (60, 61). In pigs, IFN-γ enhances production of IgG2 isotype and hence this IgG isotype is considered to be associated with Th1 immune response. On the other hand, cytokines like IL-4, IL-10 induce secretion of IgG1 and are known to be associated with Th2 immune response (62). Thus, the ratio of IgG1:IgG2 can be used to infer Th1/Th2 bias in response to vaccination. In this study, we found a higher level of IgG1 in pigs immunized with OV-HA recombinant (IgG1:IgG2 >1, Th2 bias), which suggests that the protection may have been mostly antibody-mediated in this group. Conversely, in OV-HA-NP group, the levels of IgG2 were higher (IgG1:IgG2 <1, Th1 bias), which suggests a bias towards cell-mediated immunity in this group. Given that NP protein is known to induce cell-mediated immunity, it would be safe to assume that this Th1 bias might be due to NP protein present in the OV-HA-NP recombinant.

This study further demonstrates the use of ORFV as a vaccine delivery platform in swine. The study also shows that two ORFV IMPs (ORFV121 and ORFV127) can be deleted simultaneously from the virus genome to efficiently delivery at least two viral antigens in swine. One of the advantages of ORFV-based vectors is that same vector can used repeatedly for prime boost regimens. This is important because pre-existing immunity precludes the use of many vector platforms for vaccine delivery. The humoral immune response data presented here shows that a boost effect was induced after second immunization. In fact, previous findings from our lab show that similar effect can be observed even after three immunizations with ORFV. In future, we plan to use additional immunomodulatory genes to develop ORFV as a multivalent vaccine delivery platform. We plan to use the HA gene from other IAV-S subtypes to develop multivalent vaccine candidates and evaluate heterosubtypic protection. The analysis of secretory IgA immune response, which play very important role in providing mucosal immune response is lacking in this study. Future studies involving detailed analysis of mucosal immune response and challenge with heterologous virus are warranted.

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CHAPTER 4
Identification and validation of a novel endogenous orf virus promoter using transcriptomic approach.

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Identification and validation of a novel endogenous orf virus promoter using transcriptomic approach.

Abstract

The use of poxvirus as a vaccine delivery platform has been widely studied. Orf virus (ORFV), which is a type member of parapoxvirus, has been explored as a vaccine vector particularly for use in livestock. Previous studies from our group have shown that ORFV vectored vaccine expressing spike protein of porcine epidemic diarrhea virus provides protection against PEDV in swine; and ORFV expressing hemagglutinin and nucleoprotein of swine influenza virus provides protection against swine influenza in pigs. In order to further improve the immunogenicity of ORFV vector there is a need of a promoter that leads to higher expression of heterologous proteins by orf virus. In this study, we used a transcriptomic approach to identify endogenous early/late promoters of the orf virus. To this end, the promoters of the individual open reading frames were ranked based on the number of transcripts and temporal expression profile individual open reading frame of the orf virus. Selected candidate promoters were validated using functional assays. We show that a promoter p116, which controls the expression of ORFV116 gene of ORFV is an early/late promoter. The promoter p116 leads to early and robust expression of heterologous genes. The level of protein expressed by p116 promoter is higher than that of vv7.5 promoter, which is one of the widely used vaccinia virus early/late promoter. In summary, by using transcriptomic approach we identified and validated the use of novel ORFV promoter that leads to earlier and higher expression of heterologous genes *in vitro.* The data presented here indicates that p116 can lead to enhanced expression of the heterologous genes; however further *in vivo* studies are needed to confirm the findings of this study.

Introduction

Orf virus belongs to poxviridae family. Poxviruses hold a unique place in the history of immunization. A poxvirus called vaccinia virus was used to immunize people against smallpox, which culminated with the eradication of the smallpox in 1980 (1–3). Although, smallpox was eradicated, and vaccination was discontinued, the biological and immunomodulatory properties of vaccinia virus, generated significant interest in poxviruses among scientists worldwide. Initial attempts were focused on engineering vaccinia virus to express heterologous gene (4, 5). Later, the potential of other poxviruses like avipoxvirus, parapoxvirus and swinepoxvirus to be used as vectors were also explored with promising results (6–9). Our group has been working to develop ORFV as a vaccine delivery platform for use in livestock species. Some of the features that makes ORFV as a well-received vector are: i. the large genome size, with the presence of many non-essential genes, which could be replaced with foreign gene without severely impacting virus replication; ii. the ability of ORFV to tolerate insertion of large fragments foreign DNA; iii. ability to induce both humoral and cell mediated immunity; iv. the stability of the virus at room temperature when lyophilized, which obviates the need for cold chain. We have already shown that ORFV expressing spike (S) protein of porcine epidemic diarrhea virus (PEDV) induces neutralizing antibodies in pigs and immunized pigs are protected from virulent PEDV challenge (10, 11). In another experiment, we have shown that ORFV expressing hemagglutinin gene of swine influenza virus provides protection from virulent swine influenza challenge (12). Moreover, other many groups have successfully used ORFV as a vector in swine (13, 14).

A unique feature of poxviruses is their replication site, which takes place in the cytoplasm of infected cells, making poxviruses an exception among DNA viruses (15). The virus brings its own replication machinery including RNA polymerase which obviates the need for

host RNA polymerases for replication. Most of our understanding about poxvirus replication comes from VACV (16). Notably, transcription and expression of poxviral genes is temporally regulated, and the genes are classified as early, intermediate or late genes based on the time of expression in relation to virus genome replication (17). In VACV, for example, early, intermediate and late genes are expressed in 20, 100 and 140 min after infection, respectively (18). Early genes are transcribed within the virion core soon after the virus enters the cell and mRNAs are extruded into the cytoplasm for translation. Once early genes are expressed, uncoating of the virion core takes place and DNA is released into the cytoplasm followed by viral DNA replication. The intermediate genes are expressed after DNA replication and encode for transcription factors required for the expression of late genes. The late genes encode proteins essential for virion. After virus assembly, enveloped virions are released by budding (17, 19, 20). These basic biological properties of poxviruses need to be considered when designing poxvirus vectors.

Orf virus naturally infects sheep and goat (21). We have been working to develop ORFV as a vector in pig and possibly in other species. The virus does not replicate in pig cells as efficiently as in sheep cells (12). This means ORFV should be able to express foreign gene with limited viral replication. This can only be achieved by using strong promoters. Poxvirus promoters with both early and late activity are used to ensure early and adequate expression of foreign antigens for induction of strong immune response. Some naturally occurring promoters like vv7.5 and I1L, which are present in vaccinia virus have been widely used (22, 23). In recent years, some work has been done to design poxvirus promoters using in silico method (24) . However, our previous experiments have shown that some of these vaccinia virus promoters can express heterologous gene in ORFV only after 24 hours post-infection, which severely reduces

immunogenicity of the vector. Moreover, multiple boost immunizations are required to achieve high level of foreign gene expression with these promoters. So, there is need for identification and characterization of novel promoters that could induce early and robust immune response. There is some literature that describe increase in immunogenicity of poxvirus vector by the use of native promoters (25, 26) . Which means, use of native ORFV promoter should elicit high immune response in our orf vector system instead of vaccinia virus promoters. This idea relies on the fact that these promoters must be recognized by the ORFV polymerases; a native promoter might be more efficiently bound by viral polymerase than heterologous promoters. However, there has not been any study done to identify and characterize ORFV promoters. Thus, this study aims to identify such strong early/late promoters of ORFV using transcriptomic approach that could be used to develop effective vaccine candidates.

Materials and methods

Cells and viruses

Primary ovine turbinate cells (OFTu) were cultured at 37 °C with 5% CO_2 in minimum essential medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine and containing streptomycin (100 µg/mL), penicillin (100 U/mL and gentamycin (50 µg/ mL). The ORFV strain IA82 (OV-IA82 was kindly provided by Dr. Daniel Rock at University of Illinois Urbana-Champaign).

RNA-Seq Experiment

For RNA-Seq experiment, OFTu cells were cultured in 35 mm cell culture dishes. The cells were counted and then infected with a multiplicity of infection (MOI) of 10 with ORFV-IA82 and incubated at 37 °C in the presence of 5% CO_2. For collection, cells were washed with phosphate buffer saline (PBS) and 1 ml Trizol reagent (Life Technologies) was added to the cells. The samples were harvested at 0-hour post-infection, 20 minutes post-infection, 40 minutes post-

infection, 1 hour, 2 hour, 4 hour, 6 hour, 8 hour, 12 hour and 24 hour post-infection. The lysate was stored at -80 °C until RNA extraction. Three independent replicates of the experiment were performed.

To identify early transcripts another RNA-Seq experiment was performed in the presence of cytosine-β-D- arabinofuranoside. OFTu cells were first treated with 50 µg/mL of cytosine-β-D-arabinofuranoside for 1 hour and then infected with 10 MOI of ORFV-IA82 and the cell lysates were harvested as described above.

Total RNA from the lysate was extracted following Trizol reagent protocol and purified with RNAeay RNA purification kit (Qiagen). Then, library for RNA-sequencing was prepared using TrueSeq RNA library preparation kit following the manufacturer's protocol. Sequencing was performed using Illumina HiSeq platform.

Bioinformatics data analysis

The quality of the reads was checked using FastQC (27). Low-quality reads and adapter were removed using Trim Galore (v 0.5.0) (28). A reference sequence was created by merging sheep reference genome (Oar_v4.0) with OV-IA82 genome. This reference sequence was used to align and quantify RNA-seq reads. RNA-Seq read quantification was performed using Kallisto (v 0.44.0) (29). RNA-seq reads belonging to ORFV was exported to R and all the data analysis and visualization were performed using R. The R based webtool shinyCircos was used to generate circos plot (30).

Functional validation of promoters

Functional validation of promoters was done by *in vitro* expression of green fluorescent protein (GFP). For this, plasmids containing a gene encoding GFP protein downstream of the promoter sequence were synthesized. OFTu cells were infected with 10 MOI of ORFV IA82 and

transfected with the plasmids using Lipofectamine 3000 reagent (Invitrogen). The expression of GFP protein was assessed using fluorescence microscopy and western blotting.

Western blot

Expression of GFP by different promoters was also assessed by western blot. OFTu cells infected with 10 MOI of ORFV IA82 were transfected with 2 µg of plasmid per well of a 6-well plate. The cell pellet was harvested at 1,2,4,6,8,12,24 hours post-transfection. Uninfected OFTu cells were used a negative control. Cell pellets were lysed with M-PER mammalian extraction reagent containing protease inhibitor (Thermo Scientific). Then, 50 µg of the cell lysate was resolved using SDS-PAGE in 10% acrylamide gels and transferred to nitrocellulose membrane. Blots were blocked overnight with 5% milk in PBS. The membranes were incubated with anti-gfp antibody (Santa Cruz ,cat no SC-9996) or loading control antibody for β-actin (Santa cruz, sc-8432) for 1 hour. Then, membranes were probed with Goat anti-mouse secondary antibody (LICOR, cat no: 926-32210). The membranes were visualized using Bio-Rad Chemidoc Imager.

Results

RNA-Seq experiment

The RNA-Seq experiment was run to investigate temporal expression pattern of ORFV genes. RNA-Seq was run in the OFTu cells infected with ORFV IA82 and viral transcripts were quantified (figure 4.1A). Viral transcripts were detected as early as 20 minutes post-infection and the number of viral transcripts increased with time. There was a significant increase in the number of viral transcripts after 8 hours. All the ORFV genes are expressed at high levels in 12

and 24 hours post-infection (Figure 4.1B).

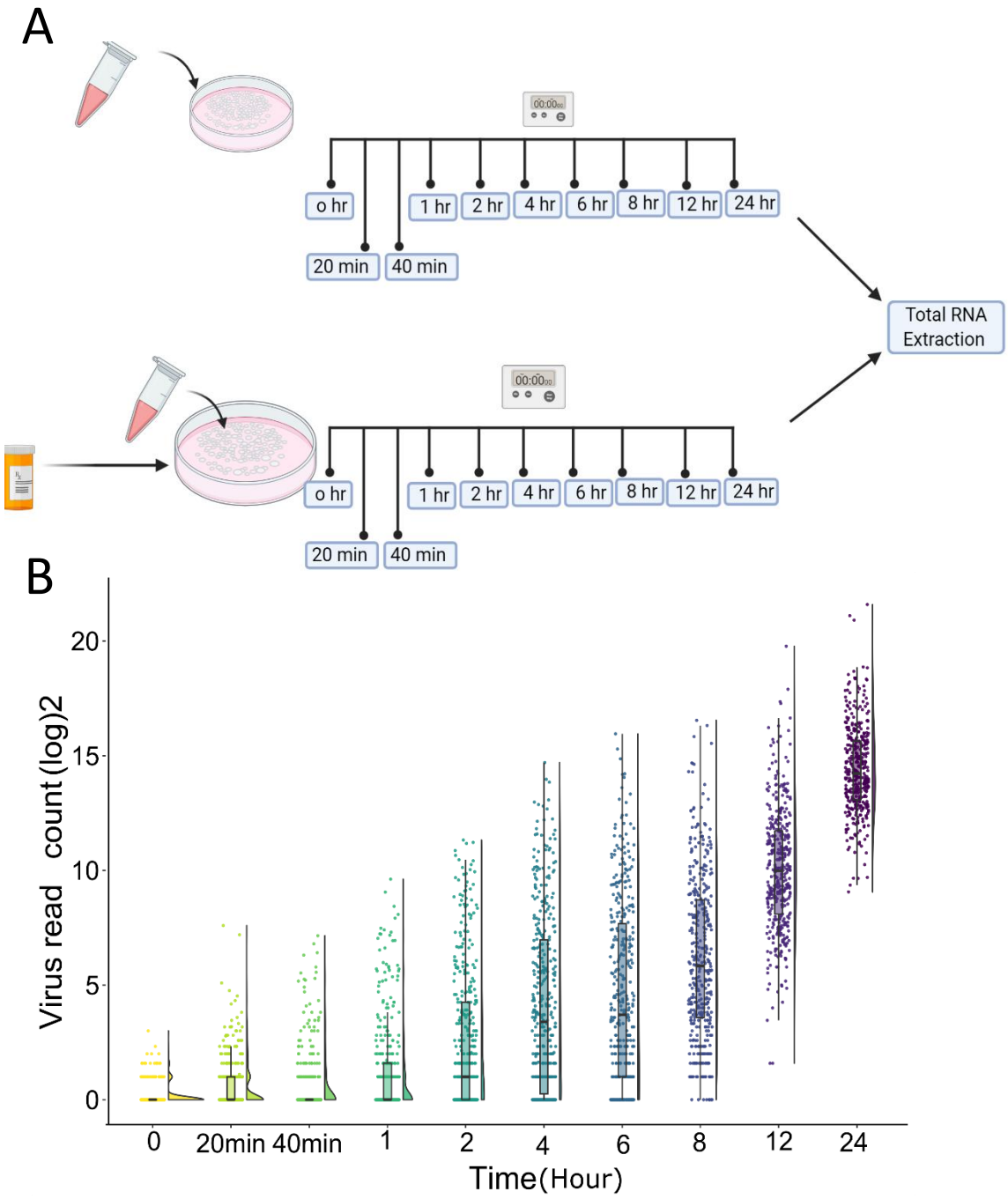

Figure 4.1. RNA-seq experiment design and read quantification. (A). RNA-seq experiment design. OFTu cells treated with or without cytosine β-D-arabinofuranoside were infected with ORFV-IA82 and harvested at different time points. Total RNA was extracted from the cell lysate and RNA-Seq was run. All the experiments were run in triplicate. (B) Total number of viral reads obtained at different time points.

Orf replication and gene expression

Next, we looked at the gene expression of the individual genes. Orf transcript were detected as early as 20 minutes post-infection (Figure 4.2). These genes that are expressed in 20 minutes are ORFV109, ORFV110, ORFV111, ORFV112, ORFV113, ORFV114, ORFV116, ORFV117, ORFV118, ORFV119, ORFV120, ORFV121, ORFV132 and ORFV134. Except for the ORFV109 and ORFV110, all the remaining genes are either involved in host-interaction or their function is unknown yet. Interestingly, these early genes maintain a high level of expression throught the replication cycle up to 24 hours post-infection. The transcripts responsible for transcription and replication can be detected at 1-hour post-infection and the levels increase with time (Figure 4.2). While most of the virion associated transcripts are detected at 12- or 24-hours post-infection, some virion associated genes are expressed 1 hpi onwards.

To determine virus replication kinetics growth curves were run on cells lysate and supernatant of the OFTu cells infected with the ORFV. The virus particles are detected in both in lysate and supernatant only at 24 hours post-infection (Figure 4.3). Overall, this data shows that viral gene expression starts within 20 minutes of infection and the virus completes its replication cycle between 12 and 24-hour post-infection.

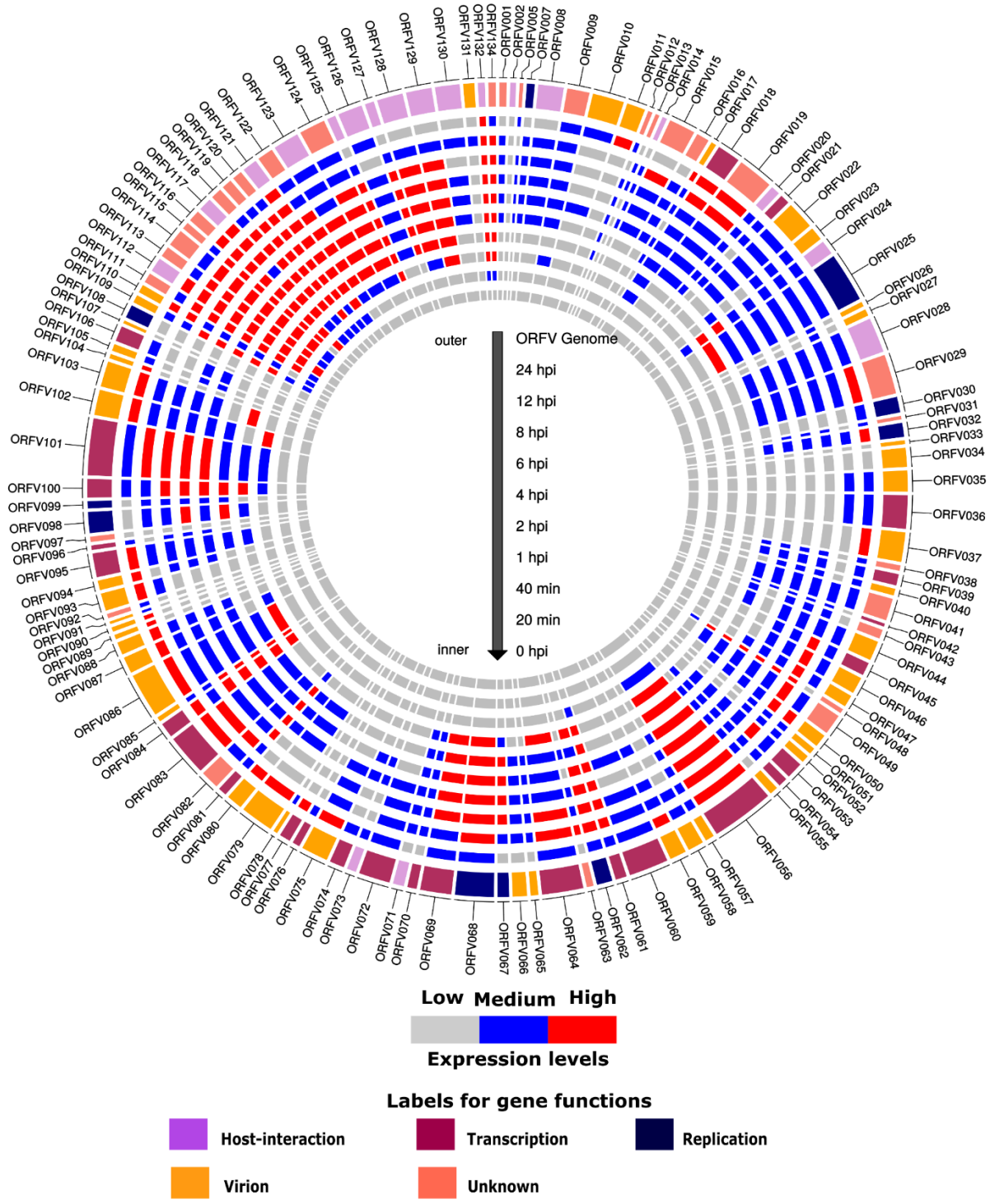

Figure 4.2 Orf virus gene expression patters. The numbers of reads corresponding to each open-reading frame of ORFV at different time-points were classified as low (less than 25 quartile), medium (between 25 and 75 quartile) and high (above 75 quartile). The outermost circle represents the orf genome with the gene color indicating their functions. The inner concentric circles represent the gene expression where the innermost circle represents 0 hpi, and other concentric circles represent the increasing time points up to 24-hour post-infection.

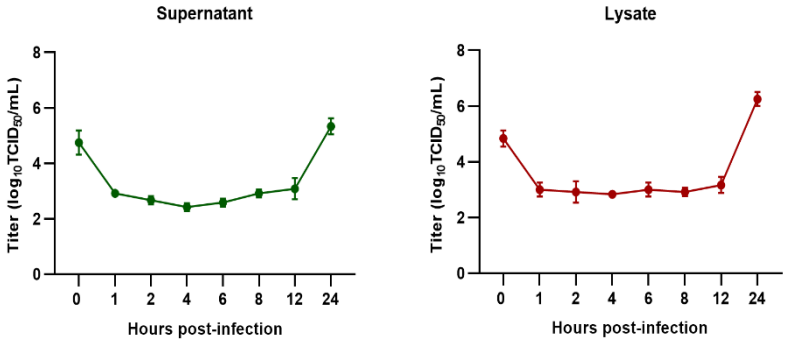

Figure 4.3 Orf virus growth curve. OFTu cells were infected with ORFV with a MOI of 10. Titrations were performed in the supernatant and the cell lysates at indicated time-points.

Identification of candidate promoters

To identify early/late promoters, we focused our assay on the genes that are expressed at very early time point and maintain their expression levels up to 24 hpi. To this end, we looked at the temporal expression pattern of the individual genes in the presence or absence of cytosine-β-D-arabinofuranoside. The drug cytosine-β-D- arabinofuranoside inhibits DNA replication so that expression of post-replicative genes are down regulated in the presence of this drug. This allows us to discriminate the early and late expression property of the genes. The expression of individual genes at different time points in the presence or absence of cytosine-β-D-arabinofuranoside is presented in Figure 4.4. The genes were ranked based on their expression profiles at different time points (data not shown). Based on the ranks, we selected ORFV112, ORFV116, ORFV119 and ORFV121, ORFV132 as the candidate promoters for further functional validation. Another gene ORFV107 was selected as a late control gene as expression of this gene is det4ected only after 12 hpi.

Functional validation of the promoters

The intergenic sequence upstream of the selected open-reading frames, namely: ORFV112, ORFV115, ORFV116, ORFV119, ORFV121, ORFV132 and ORFV107 was used as a promoter

sequence and will be referred to as p112, p116, p119, p121, p107 hereafter. Five different pUC57 plasmids containing GFP gene downstream of the selected promoter sequence were synthesized. An additional construct containing GFP under vv7.5 promoter was also synthesized, because this is an early/late promoter that has been widely used to develop poxvirus based vectored vaccines. These plasmids were transfected to the cells infected with ORFV IA82 and the expression of GFP was evaluated by fluorescence microscopy. Out of six promoters selected for functional analysis four promoters (p107, p112, p116, p119, p121) were functional as they were able to drive expression of GFP. However, one of the promoter sequence p132 failed to drive expression of GFP. Out of the five promoters that were functional two promoter p116 and p112 led to the higher expression of GFP when compared to p119 and p121. As expected, p107, which was selected as a late control gene, showed very low amount of GFP expression. The level of GFP expression by p116 and p112 was comparable to the vaccinia virus promoter vv7.5.

Comparison of temporal activity and expression levels of promoters

Based on the functional activity data we decided to further evaluate p116 and p112 promoters, since these promoters led to the higher expression of GFP in comparison to other promoters. We compared time of GFP expression and level of GFP expression by using western blot. For this assay, OFTu cells were infected with ORFV-IA82 with a MOI of 10 and then transfected with p116-GFP, p112-GFP, vv7.5-GFP and p107-GFP plasmids. The cell lysate was collected at 0,1,2,4,6,8,12,24 hours post-transfection and level of GFP was detected using a monoclonal antibody targeting GFP protein. B-actin antibody was used as a loading control.

As shown in the figure 4.7, p116 promoter led to high level of GFP expression when compared to other promoters. Also, the expression of GFP was detected 4 hours post-transfection and a high level of GFP was observed within 8 hours of transfection when cells were transfected with

p116-GFP plasmid. Another promoter p112 led to expression of GFP at 12 hours post-transfection. However, the level of GFP expression was lower than that of p116 promoter. Interestingly, p116 promoter led to higher and earlier expression of GFP than the vv7.5 promoter (Figure 4.7). No expression of GFP was detected samples transfected with p107 promoter. Thus, this result shows that p116 promoter is a strong early/late promoter that leads to early and robust expression of heterologous gene.

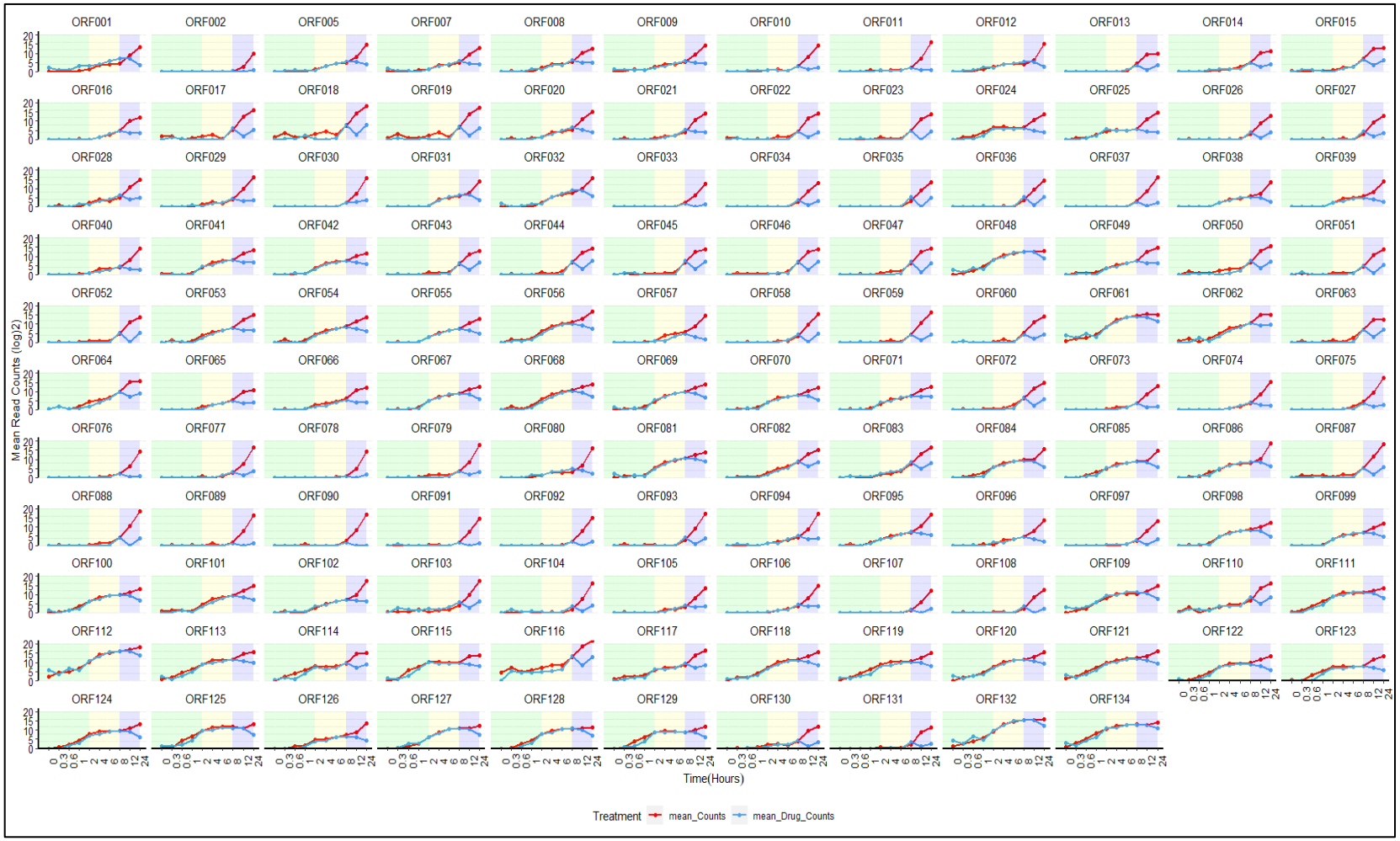

Figure 4.5 Temporal expression pattern of individual gene of the orf virus. For each graph, time points are shown in the x-axis and number of reads identified from RNA-seq (without drug- red line and with drug-blue line) and shown in the y-axis. The reads are expressed as log2.

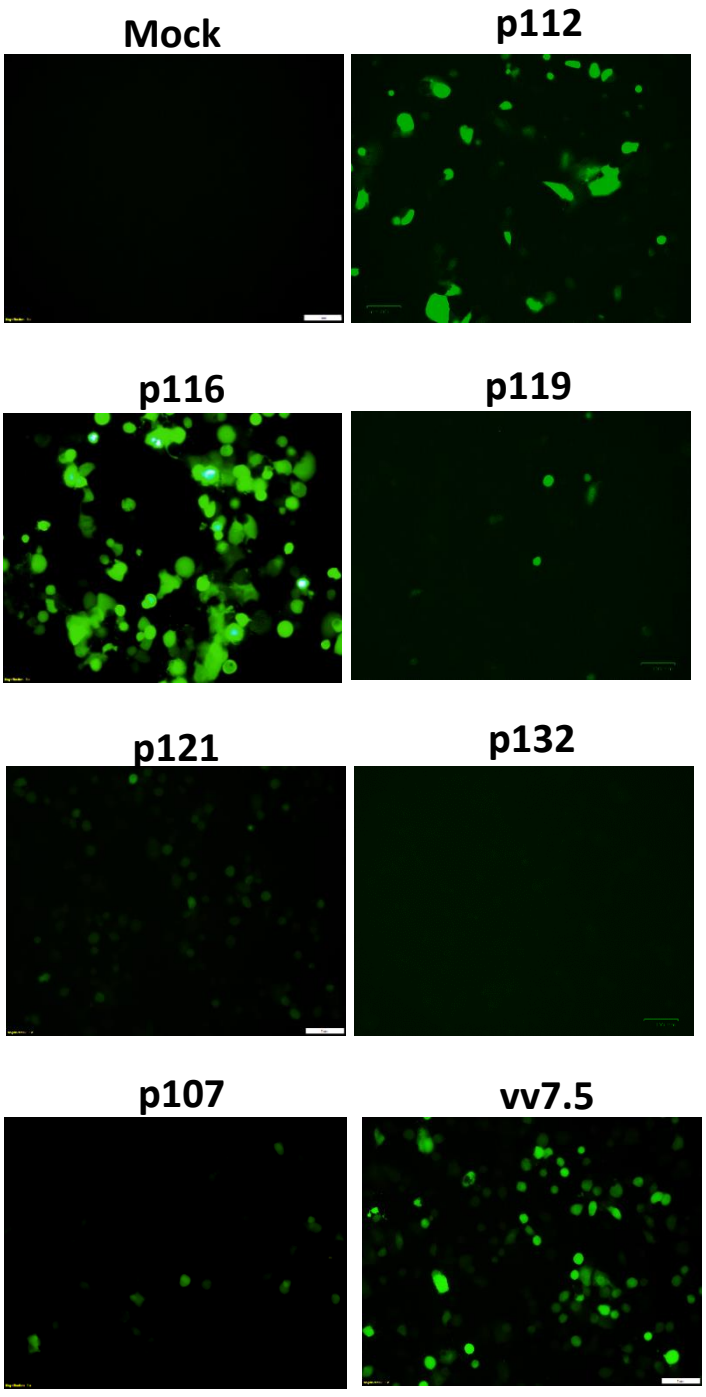

Figure 4.6 Functional validation of orf promoters. Five promoter candidates (p112,p116,p119,p121,p132) were selected based on the transcriptome analysis and GFP gene was cloned under these promoters. P107 was selected as a late control gene and vaccina virus vv7.5 promoter was used for comparison. The functional activity of the promoters was evaluated based on the expression of GFP by these promoters.

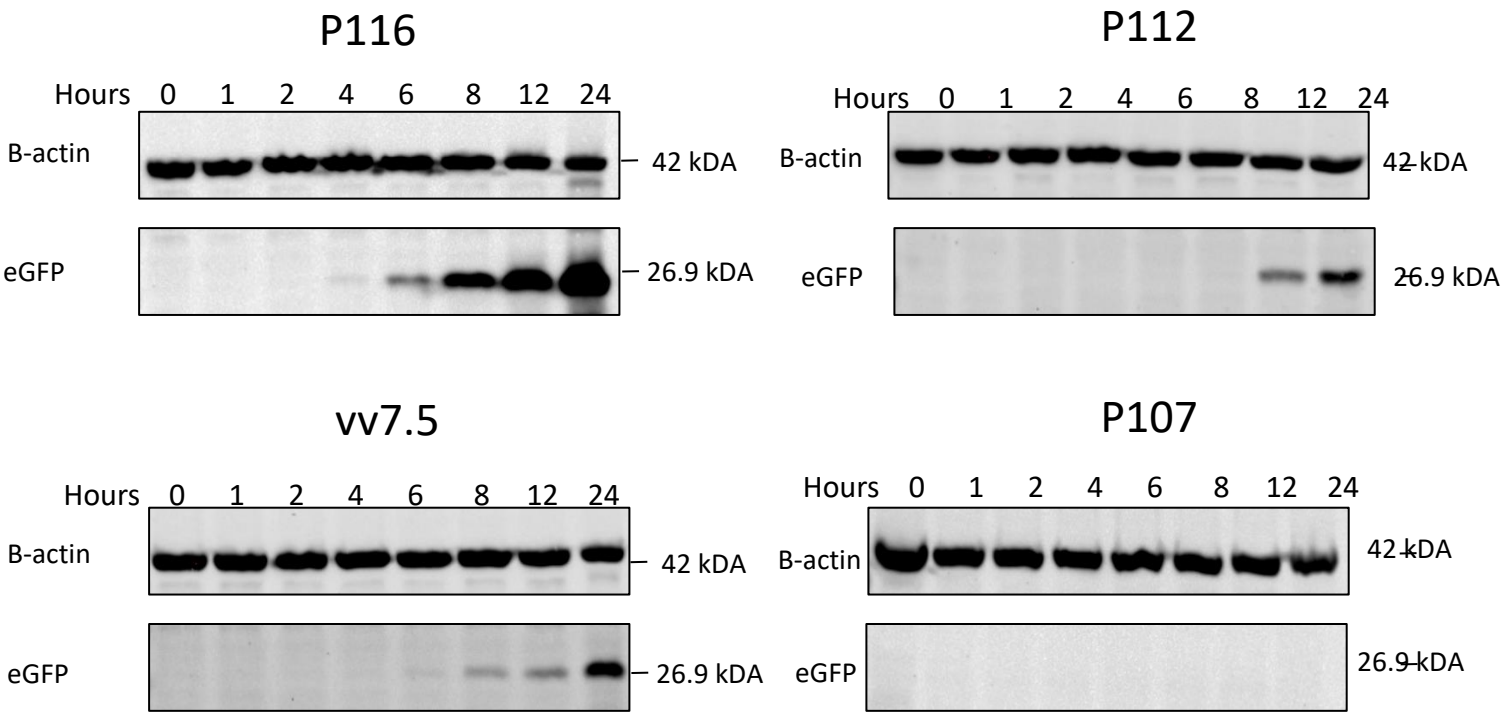

Figure 4.7. Comparison of the promoter activity. The time and levels of GFP expression by different promoters was compared using western blot. B-actin was used as a loading control.

Discussion

In this study we used transcriptomic approach to identify endogenous promoters orf virus. We identified and validated a promoter p116 which can be used to express heterologous gene in orf virus. Functional assays showed that this promoter can lead to higher level of GFP expression when compared to a widely used vaccinia virus promoter vv7.5.

This study led to comprehensive gene expression profiling of the orf genes. Although similar studies using vaccinia virus has been published before (31–33); gene expression studies for ORFV has been lacking. The data presented here would allow to understand gene expression pattern of different genes and further understand the molecular biology of the orf virus.

As shown in the figure 4.2, the ORFV expresses genes responsible for host-interaction within 20 minutes of infection. These genes play an important role in host immune evasion (34–37) although most of the genes are still not characterized, hence it is not possible to determine how these genes evade immune host immune response. Most of these genes maintain their expression throughout the replication cycles and it can be speculated that these genes are controlled by early/late promoters. The virus growth curve shows that virus completes its replication cycle between 12 and 24 hours. The new progeny virions are released between 12 and 24 hours.

Although we selected five promoters for functional analysis, only two of them p116 and p112 showed promising results. Other promoters p119, p112, p132 either failed to express GFP or expressed very low levels of GFP. This might be due to the intergenic region sequence that we selected as promoter. Some of the promoter might overlap with the neighboring open reading frame and thus we might have missed the part of the promoter sequence when limiting our selection to intergenic region. Further analysis to identify core promoter sequence would allow to identify correct position of the promoter sequence for each gene.

Overall, this study developed a comprehensive atlas of orf gene expression that can be useful to understand molecular biology of the orf virus. Most importantly, we identified a novel strong early/late promoter that could be used to express heterologous gene in ORFV or in other poxvirus-based recombinants. Since, the promoter p116 leads to very early and robust expression of heterologous gene, it would be very useful to improve the immunogenicity of orf based vectored vaccines. Further studies to evaluate immunogenicity of the recombinants developed by using p116 promoters in animal models are needed.

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CHAPTER 5
SUMMARY AND FUTURE DIRECTION

General background

Poxviruses have long been explored as a vaccine delivery vectors in human and veterinary medicine. Several poxvirus-based vector vaccines have been licensed for commercial use and many other are under clinical trials. Some important features that make poxvirus excellent candidates for vaccine delivery are: 1) Ability to accommodate large DNA insertions, 2) presence of non-essential and immunomodulatory genes which allows manipulation of the virus genome without severely impacting virus replication, 3) their stability at room temperature which obviates the need for cold chain during vaccine storage and transport, and 4) their cytoplasmic replication which decreases the risk of viral genome integration into the host genome.

This dissertation describes several studies that were devised and conducted to develop the parapoxvirus ORFV as a vaccine delivery vector for use in swine. According to the National Pork Producers Council the pork industry provides an annual gross income of nearly $20 billion to the US economy and supports about 550,000 jobs. However, the pork industry is constantly threatened by different infectious disease outbreaks that occur from time to time. Additionally, we have seen the emergence or re-emergence of several viral diseases in swine over the past few decades. This poses a significant risk to the swine industry, agriculture industry and food security. Vaccination is the most cost-effective method to prevent infectious diseases. Despite significant investments in vaccine research, vaccines are only available against a few swine diseases. In addition, protection afforded by some of the available vaccines, including SIV and porcine reproductive and respiratory syndrome virus (PRRSV), for example, is not ideal. Therefore, novel strategies and platforms for vaccine development are needed to overcome

limitations of the conventional vaccines. Thus, we decided to explore the possibility of developing ORFV as a vaccine delivery vector in swine. Orf virus has long been known for its immunostimulatory properties. Additionally, ORFV does not naturally infect swine which increases the safety profile of the virus for use in this animal species. There are several well-characterized immunomodulatory genes in the ORFV that can be targeted for rational development of ORFV-based vectors for vaccine delivery. Another important and unique property of ORFV, is the fact that the virus does not induce vector-specific neutralizing antibody responses, which allows multiple immunizations of an animal with the vector platform. These important features of the ORFV make it an attractive candidate as a vaccine delivery platform. Although some work has been done previously to develop ORFV-based vectored vaccines, extensive research particularly targeting swine species are still lacking. This work provides insights into novel strategies to develop ORFV-based vectored vaccines. The findings presented in this dissertation will have important implications on ORFV-based-vectored vaccines development in future.

Summarized findings

Chapter 1 of this dissertation provides a comprehensive review on the use of poxvirus vectors in the veterinary field. The literature related to the different poxvirus vectors and how they have been used to develop either experimental vaccines or commercial vaccines have been reviewed and the main finding summarized. This chapter also includes important information about the molecular biology of poxvirus and theoretical aspects related to poxvirus based vectored vaccine development.

In Chapter 2, we investigated whether a recombinant ORFV expressing the full-length spike (ORFV-PEDV-S) can induce maternal immunity to porcine epidemic diarrhea virus (PEDV) in

pigs. The results presented in chapter two show what when pregnant sows are immunized with ORFV-PEDV-S, they seroconvert and develop spike-specific IgG and IgA antibodies that can be detected in serum and in milk/colostrum after farrowing. We demonstrated that spike specific antibodies are transferred to the piglets via milk and colostrum. Most importantly, piglets born from OV-PEDV-S immunized sows had significantly lower mortality when compared to the piglets born to sham-immunized sows after challenge with a virulent PEDV strain.

In Chapter 3, we developed ORFV recombinants expressing either hemagglutinin (OV-HA) or hemagglutinin and nucleoprotein (OV-HA-NP) of the swine influenza virus (IAV-S) and evaluated the immunogenicity and protective efficacy of these recombinants in pigs. Both OV-HA and OV-HA-NP recombinants elicited robust neutralizing antibody response in pigs. Both recombinants elicited IAV-S-specific T-cell response; however, T-cell response induced by OV-HA-NP was comparatively higher than that of OV-HA. Importantly, pigs immunized with either OV-HA or OV-HA-NP had reduced virus shedding in nasal swabs, reduced viral load in the lung and the immunized pigs were protected against lung lesions upon challenge infection when compared to the sham-immunized control animals.

In Chapter 4, we used a transcriptomics approach to identify novel early/late promoters of the ORFV in an attempt to enhance expression and delivery of heterologous genes by ORFV-based vectors. Five candidate promoters were selected based on the transcription profile. These promoters were characterized by cloning the green fluorescent gene under these candidate promoters. The results presented in the chapter 3 show that an ORFV promoter that drives the expression of the ORFV116 gene is an early/late promoter and it leads to the robust expression of the heterologous gene by ORFV vectors. Additionally, the transcriptomic data presented in this chapter provides gene expression profiles for individual genes which could be useful to

understand the molecular biology of the ORFV.

Future direction

This dissertation provides evidence that ORFV is a promising vector candidate for vaccine delivery in swine. We used two well-characterized ORFV loci (ORFV121 and ORFV127) to develop vectored-vaccine against porcine epidemic diarrhea virus (PEDV) and swine influenza virus (IAV-S). However, there are many other immunomodulatory proteins that are encoded by ORFV which could be further explored to improve the immunogenicity of the ORFV. Previous work from our lab has shown that choice of insertion sites has a significant impact on the immune response elicited by the ORFV recombinants. Hence, studies to assess the effect of deletion of other immune-modulating genes like ORFV112, ORFV073, ORFV002, ORFV117 on the immunogenicity and safety of ORFV are highly desirable. Furthermore, this would also allow us to develop multivalent vaccines targeting more than one disease by providing additional loci for heterologous gene insertion.

Our work has shown that ORFV-PEDV-S recombinant induces maternal immunity in pregnant sows. However, the underlying mechanism of how ORFV-PEDV-S induces spike specific IgG and IgA antibody response in milk is still not completely understood. It has been widely accepted that antigenic stimulation in the gut is required to induce PEDV specific antibodies in the milk. Previous studies have shown that upon antigenic stimulation of immunocytes in the intestines they move to the mammary gland via the gut-mammary axis. It has also been reported that homing receptors like MaDCAM-1, α4β7 are important in mediating the transfer of plasmablasts from gut-associated lymphatic tissues to the mammary gland. However, the mechanism by which animals that are immunized parenterally with ORFV-PEDV-S induced spike specific antibodies in milk and colostrum remains to be explored. One possibility is that dendritic cells might be

responsible for carrying antigens from the injection site to the gut lymphoid tissues to mimic what usually happens during natural infection. On the other hand, it is likely that serum antibodies could be the direct source of IgG and IgA antibodies present in the milk. To understand this mechanism experiments to measure PEDV specific antigen secreting cells (ASC) in the gut, mammary gland and blood after ORFV-PEDV-S immunization are required.

Our OV-HA and OV-HA-NP recombinants induce a high level of neutralizing antibodies in pigs and animals are also protected from the experimental challenge. The vaccine candidates developed here targets the H1N1 subtype. The prevalence of other subtypes mainly H1N2 and H3N2 is increasing in the US. Hence using hemagglutinin from other subtypes to develop a multivalent vaccine targeting all three subtypes will have huge implications in developing a vaccine candidate that can be used in the field conditions. Our work has shown that these ORFV-based vaccine candidates induce both humoral and cell-mediated immune responses. Several studies have highlighted the importance of mucosal immune response in protecting against influenza viruses. Hence, it would be interesting to assess if OV-HA or OV-HA-NP induce an influenza-specific mucosal immune response in pigs. If these ORFV recombinants are able to elicit a mucosal immune response, it might be possible that ORFV-based vectored vaccines targeting several other respiratory or enteric pathogens could be developed and their protective efficacy can be evaluated in future.

We have shown that a novel ORFV promoter P116 leads to an earlier and higher level of transgene expression. This result is based on the expression of green fluorescent protein. Now, it is imperative to test this promoter using an antigen from a pathogen (for example hemagglutinin (HA) of IAV-S) and then develop recombinants using this promoter. In vitro experiments designed to perform a head-to-head comparison of P116 promoter with other vaccinia virus or

orf virus promoters are needed to confirm the findings of this study.

One of the bottlenecks in developing ORFV recombinants is the time it takes to obtain a pure recombinant. ORFV recombinants are developed by homologous recombination which is a highly inefficient process and thus requires several rounds of plaque assay to obtain a recombinant. Due to the prohibitively high cost of gene synthesis, developing synthetic poxvirus was not feasible previously. However, the cost of gene synthesis has decreased over the last years. Thus, it is possible to synthesize the full genome of poxvirus and develop recombinants rapidly without the need for an arduous selection process. A few synthetic poxvirus recombinants have already been developed by different labs which have provided a proof-of-concept that synthetic poxviruses can be used to develop a poxvirus recombinant successfully. Thus, future work to establish synthetic ORFV would make developing ORFV recombinants more convenient. In addition, having a synthetic ORFV will also help to develop vaccines in response to pandemics as this system would allow generating ORFV recombinants rapidly.

In conclusion, this dissertation demonstrates the applicability of ORFV as a vaccine delivery platform for use in swine. The results presented here will have important implications in the rational development of ORFV vectored vaccines against different viral diseases of swine.