

Broadly protective Bovine Parainfluenza-3 Virus and Bovine Viral Diarrhea Virus vaccine

by

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AN ABSTRACT OF A DISSERTATION

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Department of Diagnostic Medicine and Pathobiology
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Abstract

Bovine Parainfluenza-3 virus (BPI3V) and Bovine Viral Diarrhea Virus (BVDV) are major respiratory pathogens contributing to Bovine Respiratory Disease Complex, leading to significant economic losses. While good management practices and biosecurity measures are important, vaccination remains the most effective control strategy. However, existing commercial BPI3V vaccines, based on genotype A strains, confer protection against some, but not all, genotype A strains and elicit low neutralizing titers against genotypes B and C. Similarly, current commercial BVDV vaccines, formulated using BVDV-1a and BVDV-2a strains, confer partial protection against strains from homologous genotypes and low neutralization titers against heterologous and emergent genotypes. This Thesis presents studies aimed at developing a live vaccine capable of inducing broad protection against BPI3V and BVDV strains. In the first study, a BPI3V vaccine vector based on a genotype C strain was developed and validated for *in vitro* attenuation, transgene expression, and genetic stability. Novel mosaic BPI3V Fusion (F2) and Hemagglutinin-Neuraminidase (HN2) antigens, derived from consensus protein sequences of BPI3V genotypes A, B, and C, were used to develop a recombinant prototype vaccine, designated rBPI3V_{cmut}F2HN2. Calves vaccinated intranasally with the rBPI3V_{cmut}F2HN2 vaccine exhibited robust systemic and mucosal IgG responses against all the three BPI3V genotypes, which were significantly amplified after boosting. Notably, the prototype vaccine induced significantly higher ($p < 0.0001$) neutralizing antibodies against BPI3V genotypes A-C compared to a commercial vaccine, and the robust IgG responses were primarily primed and expanded by the mosaic F2HN2 antigens. Following challenge with wild-type BPI3V genotype C virus, the rBPI3V_{cmut}F2HN2-vaccinated calves shed the least amount of virus in nasal fluids, had lower viral loads in their blood, and exhibited minimal pulmonary lesions. These findings demonstrate that the live

rBPI3Vc_{mut}F2HN2 prototype vaccine is a promising candidate for inducing broad BPI3V protection compared to the current commercial vaccine. In the second study, a BPI3Vc-vectored BVDV prototype vaccine was developed using novel mosaic BVDV E2-NS2-5¹⁻² antigens. These antigens, designed from the consensus of all BVDV-1a, -1b, -2a, -2b, and -2c protein sequences, were used to generate recombinant BPI3Vc_{mut}E2-NS2-5¹⁻² viruses, collectively designated as rBPI3Vc_{mut}BVDV. Intranasal immunization of calves with the rBPI3Vc_{mut}BVDV prototype vaccine elicited higher IgG responses against BVDV-1b California and TGAC, with significantly higher viral neutralizing titers (California: $p=0.0031$, TGAC: $p=0.0002$) compared to a commercial vaccine. Immunized calves also elicited significantly higher VN titers against BVDV-2a strains 296NC ($p=0.0006$), 890 ($p=0.0020$), 296C ($p=0.0464$), A125 ($p=0.0018$), and 1373 ($p=0.0025$). Following challenge with BVDV-1b California, the rBPI3Vc_{mut}BVDV vaccinees exhibited a steady weight gain, lower lymphopenia, lower blood viral load, and fewer gross lesions. Overall, data generated from this thesis support using the live-attenuated BPI3Vc-vector for development of contemporary broadly protective BPI3V and BVDV vaccines that can easily be upgraded for improved disease management and cattle productivity.

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Dedication

I dedicate this work to my Chigil and Bartai family, your inspiration has shaped this journey. To Dad, Mum, Grace, Judy, Brian, Nathan, Betu, and my husband Moses, your love and support has made this adventure possible. Thank you.

Chapter 1 - Latest Advances in Next-Generation BPI3V and BVDV

Vaccines

1.1 Bovine Respiratory Disease Complex (BRDC)

Bovine Respiratory Disease Complex (BRDC) is a multifactorial respiratory condition significantly affecting cattle health and productivity. The etiologic agents responsible for BRDC are a combination of bacterial and viral pathogens, including Bovine Parainfluenza Virus (BPI3V) and Bovine Viral Diarrhea Virus (BVDV) (Bell, Turkington, & Cosby, 2021; Grissett, White, & Larson, 2015), along with non-pathogenic factors such as transportation or environmental stressors (Taylor, Fulton, Lehenbauer, Step, & Confer, 2010). Acute BPI3V infections can lead to respiratory disease, presenting symptoms such as coughing, fever, and nasal discharge (Caswell & Williams, 2016; J. A. Ellis, 2010). BVDV infection is associated with diarrhea, respiratory complications, and reproductive losses (Houe, 2003). BRDC poses a major economic burden on the cattle industry, which contributes over \$ 100 billion annually to the U.S. economy (USDA, 2023). This leads to significant financial losses due to increased cost of treatment, decreased productivity, and mortality rates of up to 70% (Johnson & Pendell, 2017; Loneragan, Dargatz, Morley, & Smith, 2001). Given its economic impact, BPI3V and BVDV are High-Priority diseases in animal agriculture. Developing effective vaccines capable of reducing morbidity, mortality, and antibiotic reliance is essential for improving the efficiency and sustainability of beef and dairy production.

1.2 Bovine Parainfluenza -3 Virus (BPI3V)

Bovine parainfluenza-3 Virus (BPI3V) is a respiratory pathogen first isolated in 1958 in the United States from cattle exhibiting signs of shipping fever and was initially referred to as myxovirus SF-4 (J. A. Ellis, 2010; Reisinger, Heddlestone, & Manthei, 1959). The virus is an enveloped, single-stranded, negative-sense RNA virus belonging to the genus *Respirovirus* within the family *Paramyxoviridae*. Its genome, approximately 15.5 kb in length, encodes six structural proteins, including Nucleoprotein (N), Phosphoprotein (P), Matrix (M), Fusion (F), Hemagglutinin-Neuraminidase (HN), and Large/polymerase (L). Three non-structural proteins (V, C, and D) result from RNA editing of the P gene or insertion of extra guanine (G) residues, generating four open reading frames (Fig. 1.1) (J. A. Ellis, 2010; Galinski, Troy, & Banerjee, 1992). BPI3V is classified into three genotypes (A, B, and C) based on phylogenetic and antigenic differences (R. Fulton et al., 2017; John D Neill, Ridpath, & Valayudhan, 2015). Genotype A has been identified in the United States, Australia, China, Argentina, Japan, and Egypt (Bailly, Mcauliffe, Skiadopoulos, Collins, & Murphy, 2000; Horwood, Gravel, & Mahony, 2008; Leal et al., 2019; Maidana et al., 2012; John D Neill et al., 2015; Ohkura, Kokuho, Konishi, Kameyama, & Takeuchi, 2013; Sobhy et al., 2017). Genotype B, initially isolated in Australia, has been detected in the United States, Argentina, and China (Horwood et al., 2008; Maidana et al., 2012; John D Neill et al., 2015; X. Wang et al., 2022). Genotype C has been reported in the United States, China, South Korea, and Japan (Konishi et al., 2014; John D Neill et al., 2015; Oem et al., 2013; Zhu et al., 2011). Effective control of BPI3V relies on systematic prevention strategies such as strict biosecurity measures, stress reduction, good management practices, and vaccination. Among these, vaccination remains the most effective tool for controlling BPI3V (Hjerpe, 1990).

1.2.1 Current BPI3V vaccines

The first BPI3V vaccines were developed from the SF-4 strain (Genotype A) shortly after its discovery as Modified Live Virus vaccines (MLVs) (26, 27) or Killed Virus vaccines (KVs) (B Morein, 1972; Probert, Stott, Thomas, Collins, & Jebbett, 1978). Since then, several commercially available MLVs have been developed as temperature-sensitive mutants (Bryson et al., 1999; Vangeel, Ioannou, Riegler, Salt, & Harmeyer, 2009; Zygraich, 1979). Current vaccines mainly include multivalent formulations administered along with one or more pathogens or bacteria associated with BRDC, such as Bovine Viral Diarrhea Virus (BVDV) (R. Fulton et al., 2016; Masic et al., 2020). MLVs contain attenuated BPI3V strains that can replicate in the host, eliciting humoral and cell-mediated immunity and effectively protecting against BPI3V infection (J. A. Ellis, 2010; Flynn et al., 2024; Masic et al., 2020; Vangeel et al., 2009). While MLVs are administered intranasally or parenterally, intranasal vaccination is more effective in stimulating nasal mucosal antibody responses, the site of pathogen entry and pathogenesis (J. A. Ellis, 2010; Todd, 1975; Woods, Crandell, & Mansfield, 1975). Intranasally administered temperature-sensitive BPI3V vaccines have restricted replication within the lower respiratory tract, which is a safety feature (J. A. Ellis, 2010; Vangeel et al., 2009; Zygraich, 1979). Vaccination against BPI3V helps reduce viral shedding, lowers morbidity, and decreases the risk of secondary bacterial infections associated with BRDC (Hjerpe, 1990; Masic et al., 2020; van Rooij et al., 2023; Vangeel et al., 2009).

1.2.2 Immunity elicited by BPI3V vaccines

Maternal antibodies transferred via colostrum provide the initial protection against BPI3V infections needed by neonates and yearlings (Dawson, 1966; Marshall & Frank, 1975). As

maternal antibodies wane, vaccination is needed to confer protection. Intranasal immunization stimulates both local and systemic neutralizing antibody responses. Mucosal antibodies are mainly associated with preventing infection, while serum antibodies reduce disease severity after infection occurs (Caldow et al., 1993; Gates et al., 1970; Marshall & Frank, 1971). Intranasal vaccination has been shown to elicit protective neutralizing antibodies in calves as young as 3 to 8 days old (Wenzhi Xue et al., 2010). Similarly, parenteral inactivated vaccines, which were initially demonstrated to induce both systemic and local antibodies in seronegative calves (B Morein, 1972; Probert et al., 1978), also elicits neutralizing antibodies as early as two weeks after vaccination in 6- to 9-month-old calves (El-Bagoury, El-Nahas, Mansour, & Kassem, 2015). A live bivalent intranasal vaccine has been shown to prime BPI3V-specific neutralizing antibodies even in the presence of maternally derived antibodies, with a recall response detectable upon challenge 92 days later (Metcalf et al., 2020). Another live vaccine has been shown to activate mucosal innate immune responses and upregulate genes involved in innate, antiviral, and Th1-mediated immunity (CXCL10, CCR5, IFN β 1, IL-18, and IFN- γ), leading to an early onset of protection (within seven days) in newborn colostrum-deprived calves (Nuijten et al., 2022). Notably, this live vaccine can be administered on the day of birth, conferring early protection against BPI3V infection (van Rooij et al., 2023). Natural Killer (NK) cells and cytotoxic T lymphocytes (CTLs) have been shown to exhibit cytotoxic activity against BPI3V 6 to 9 days after infection (Bamford, Adair, & Foster, 1995). In the closely related Sendai virus model, CD8⁺ CTLs increase 5 to 7 days post-infection and play a crucial role in viral clearance and resolution. However, the cytotoxic activity of CD8⁺ CTLs is relatively short-lived and weaker compared to the long-lasting protection conferred by virus-neutralizing antibodies (Tao et al., 2000).

1.2.3 Limitations associated with current BPI3V vaccines

Although live attenuated and killed BPI3V vaccines have been in use for decades, their protective efficacy remains a concern due, in part, to antigenic variability. One key issue is that vaccine strains differ from circulating field strains, making commercially available vaccines effective against some, but not all, homologous and heterologous strains. In the United States, vaccine formulations have traditionally relied on live attenuated BPI3V genotype A strains, including SF-4 and Kansas /15626/84 strains (Bailly et al., 2000; Bryson et al., 1999; R. Fulton et al., 2016; John D Neill et al., 2015; Thorsen, Sanderson, & Bittle, 1969). However, recent studies indicate that, in addition to genotype A, genotype B and C strains are also widely circulating in the U.S. (R. Fulton et al., 2017; John D Neill et al., 2015; Benjamin W Newcomer et al., 2017). Importantly, reference sera from SF-4 genotype A neutralized some, but not all, genotype A strains and showed lower titers against genotype B and C strains (John D Neill et al., 2015). A Viral Neutralizing (VN) titer of 1:32 is considered protective against BPI3V (J. A. Ellis, 2010). Therefore, neutralizing titers elicited by the reference sera in some cases fell below the protective threshold of 1:32, highlighting the genetic and antigenic variability of field strains (John D Neill et al., 2015). This variability may increase the risk of infection and morbidity in cattle exposed to emerging BPI3V variants.

Some vaccines, including recently developed commercial formulations in the U.S., are administered parenterally (El-Bagoury et al., 2015; Masic et al., 2020). However, since peak BPI3V infection occurs within the first month of life, vaccines should ideally be administered within the first few weeks (Lemon & McMenemy, 2021). In the presence of maternal antibodies, parenteral vaccines are more vulnerable to neutralization and complement-mediated clearance that limit its efficacy (J. Ellis, K. West, V. Cortese, C. Konoby, and D. Weigel., 2001; R. W. Fulton, J. F. Ridpath, S. Ore, A. W. Confer, J. T. Saliki, L. J. Burge, and M. E. Payton., 2005.; Zimmerman,

Boots, Valli, & Chase, 2006). Additionally, parenteral administration does not mimic the natural route of BPI3V infection, reducing its effectiveness in eliciting robust mucosal immune responses (R. Fulton et al., 2017).

Another concern with MLVs is the potential for reversion to virulence and viral shedding. Presence of an adventitious virus can result in viral shedding by vaccinated calves, potentially compromising disease control efforts and contributing to viral spread (56). On the other hand, inactivated vaccines, while considered safer, require multiple doses and may fail to induce strong mucosal immunity (Lemon & McMenamy, 2021).

1.2.4 Protective BPI3V antigens

The Fusion (F) and Hemagglutinin-Neuraminidase (HN) glycoproteins are the primary immunodominant antigens responsible for eliciting neutralizing antibody responses (Collins, 1996). Early studies demonstrated that calves develop serum antibodies against F and HN within one week of exposure to BPI3V aerosols (T. Toth & Frank, 1988). Upon re-exposure, a strong recall response is elicited, and serum antibody titers against these antigens correlate with protection (T. Toth & Frank, 1988). In humans infected with Parainfluenza virus, it was observed that while serum antibodies were generated against multiple viral proteins, only those against F and HN were capable of neutralizing wild-type viruses *in vitro* and *in vivo* (Kasel, Frank, Keitel, Taber, & Glezen, 1984). Initial viral infection elicits higher HN-specific titers, whereas re-infection boosts F-specific titers (van Wyke Coelingh et al., 1990), highlighting the benefits of a prime-boost regimen in experimental vaccine studies. Given the critical role of F and HN as vaccine targets, numerous vaccine candidates have been developed and tested in cattle, including recombinant BPI3V protein subunit, DNA, nanoparticle-based, and viral-vectored vaccines.

1.2.5 Recombinant BPI3V protein subunit vaccines

The F and HN antigens, when formulated as 30S protein micelles at doses of 1 µg or 10 µg, elicited IgG titers of 40 µg/mL and 90 µg/mL, respectively, following parenteral prime-boost immunization of mice (Bror Morein, Sharp, Sundquist, & Simons, 1983). When formulated with an oil-based adjuvant, this immunogen successfully induced antibody responses that protected lambs against PI-3-induced pneumonia (Bror Morein et al., 1983). Additionally, a baculovirus-expressed HN antigen was shown to elicit serum-neutralizing antibodies in colostrum-deprived calves following parenteral immunization and, unexpectedly, mucosal antibodies, which contributed to protection upon challenge (Haanes, Guimond, & Wardley, 1997).

1.2.6 DNA vaccines

DNA plasmids encoding F and HN antigens under the control of human Cytomegalovirus promoter have been shown to elicit high serum antibody responses following intradermal immunization in mice, whereas intramuscular immunization primarily stimulated antigen-specific, Th1-biased T cell proliferation. In cotton rats, both intradermal and intramuscular immunization routes conferred protection against BPI3V challenge (Braun, Karvonen, King, Yoo, & Babiuk, 1999).

1.2.7 Nanoparticle-based vaccines

Nanoparticles enhance antigen stability and promote a targeted immune response. In mice, intranasal immunization with BPI3V-solubilized antigens encapsulated in poly(dl-lactic-co-glycolide) (PLGA), but not an encapsulated BPI3V peptide motif, elicited an early IgG response that persisted for six weeks, suggesting a slow and continuous antigen release (Mansoor et al., 2014). In cattle, a BPI3V antigen preparation encapsulated in PLGA induced significant BPI3V-

specific mucosal IgA responses in intranasally immunized calves with pre-existing maternal antibodies. These mucosal IgA levels were strongly recalled following a booster dose and persisted until the end of the 92-day study (Mansoor et al., 2015). The vaccine also primed BPI3V-specific IgG responses, which were recalled after a single boost and remained elevated throughout the study, which was comparable to a commercial vaccine (Mansoor et al., 2015). Vaccination had no significant impact on cell-mediated immune responses (Mansoor et al., 2015).

1.2.8 Live vectored vaccines

Two concurrent studies evaluated the immunogenicity and efficacy of a recombinant replication-incompetent human Adenovirus 5 (HAd5)-vectored BPI3V vaccine against BPI3V genotype C. In the first study, a recombinant HAd5 vector expressing the BPI3V-C F antigen induced neutralizing antibody titers of 1:256 in intramuscularly immunized mice, which were significantly higher than those observed in intranasally immunized mice (1:128) or mice receiving an inactivated BPI3V-C vaccine (1:108) (J. Zhang et al., 2023). Intramuscular immunization also increased CD4⁺/CD8⁺ T cells in the spleen and enhanced IL-4 and IFN- γ cytokine responses, contributing to reduced viral load upon BPI3V-C challenge (J. Zhang et al., 2023).

In the second study, a recombinant HAd5 construct expressing both BPI3V-C F and HN antigens elicited higher neutralizing antibody titers (1:640) against BPI3V-C in mice following intramuscular immunization, along with an increase in splenic CD3⁺ and CD8⁺ T cell percentages and IL-4 cytokine expression, leading to a significantly lower viral load upon challenge (J. Zhang et al., 2024). In calves, intramuscular vaccination induced BPI3V-C-specific (SMU-SC20) neutralizing antibodies, with Viral Neutralizing (VN) titers of 1:426 against BPI3V-C and VN titers of 1:85 against the BPI3V-genotype A strain (B15) (J. Zhang et al., 2024).

1.2.9 Conclusion

Despite the availability of Modified Live and Killed BPI3V vaccines, BPI3V infections remain a leading cause of pneumonia, morbidity, and mortality in cattle. There is a widening gap in genetic and antigenic variability. Consequently, current vaccines lack sufficient efficacy to provide cross-protection against diverse BPI3V strains in North America. While recent vaccine efficacy studies offer valuable insights for improving vaccine formulations, more effective, long-lasting vaccines that provide broad protection are needed to enhance BPI3V disease management and reduce economic losses in the cattle industry.

1.3 Bovine Viral Diarrhea Virus (BVDV)

Bovine Viral Diarrhea Virus (BVDV), a *pestivirus* in the family *Flaviviridae*, is a pathogen that affects cattle globally (Becher et al., 2003; Scharnbock et al., 2018; Smith et al., 2017). The BVDV polyprotein, encoded by a 12.5 kb viral RNA genome, undergoes proteolytic processing resulting in four structural proteins (Capsid, E^{ns}, E1, and E2) and seven non-structural proteins (N^{pro}, p7, NS2-3, NS4A-B, and NS5A-B) (Fig. 1.2) (J. D. Neill, 2013). In cattle, BVDV infects the gastrointestinal, respiratory, and reproductive tracts, causing a wide range of symptoms, including fever, diarrhea, pneumonia, congenital defects, and abortion (Ackermann, Derscheid, & Roth, 2010; J. Ridpath, 2010). The virus is highly virulent and spreads within herds through persistently infected (PI) animals (Chernick et al., 2018; R. W. Fulton, 2013). Additionally, BVDV is immunosuppressive, making infected cattle more susceptible to opportunistic secondary infections, often leading to bovine respiratory and diarrhea disease (BR/DD) (J. Ridpath, 2010). Due to its high morbidity and mortality rates, BVDV infection results in significant economic losses for the dairy and beef industries (Givens & Newcomer, 2015; Johnson & Pendell, 2017).

BVDV is an extremely diverse pathogen classified into two genotypes, *Pestivirus* A and B (previously known as BVDV-1 and -2). These genotypes are further divided into 22 BVDV-1 sub-genotypes (BVDV-1a to -1v) and four BVDV-2 sub-genotypes (BVDV-2a to -2d) (ICTV; Kalaycioglu, 2007; Yesilbag, Alpay, & Becher, 2017). Some BVDV strains exhibit a lytic effect on infected cells and are classified as either cytopathic (cp) or non-cytopathic (ncp) strains (J. F. Ridpath, 2005; Tian et al., 2021; Yesilbag et al., 2017).

1.3.1 Current BVDV Vaccines

The first BVDV vaccines were developed nearly sixty years ago with the attenuation of the BVDV-1a Oregon C24V strain (Coggins et al., 1961; van Oirschot, 1999). Since that time, both Modified Live Virus vaccines (MLVs) and Killed Virus vaccines (KVs) have been available for commercial use (Griebel, 2015; Masic et al., 2021; Newcomer, Chamorro, & Walz, 2017). In North America and parts of Europe, vaccination, alongside biosecurity measures such as identifying and eliminating persistently infected (PI) animals, are the main strategies for BVDV control (Arnoux et al., 2021; Brock, 2004; Scharnbock et al., 2018; Sozzi et al., 2020). Multivalent MLVs and KVs are formulated based on the prevalent sub-genotype(s) and contain two or more BVDV strains (Robert W Fulton, Cook, Payton, Burge, & Step, 2020; Sozzi et al., 2020). The efficacy of current BVDV vaccines depends on the diversity of endemic strains and the strain composition present in the vaccines (Robert W Fulton et al., 2020; Klimowicz-Bodys et al., 2022; J. F. Ridpath, 2005; Sozzi et al., 2020). BVDV vaccination is mainly aimed at preventing the birth of PI calves, decreasing viremia, alleviating morbidity, and protecting against BRD (Arnoux et al., 2021; B. W. Newcomer et al., 2017; Newcomer, Walz, Givens, & Wilson, 2015).

An attenuated BVDV with defined mutations is considered a safer MLV vaccine than a spontaneously mutated attenuated BVDV. In this regard, attenuated BVDV mutants from wild-type BVDV strains have been generated by genetic modification via deletions within the 5' untranslated region (UTR), N^{pro}, Capsid, E^{ms}, and E1 genes. The BVDV vaccines generated by targeted gene deletion have been demonstrated to be stable, highly immunogenic, and efficacious against multiple BVDV strains in cattle. N^{pro} and E^{ms} gene-deleted BVDV mutants do not infect *in utero* fetus to develop persistent infections, and they induce higher levels of type 1 IFN (Carlson et al., 2021; Makoschey et al., 2004; Meyers et al., 2007; Wernike et al., 2018; Zemke, König, Mischkale, Reimann, & Beer, 2010). A vaccine containing BVDV-1 and -2 mutants that lack functional N^{pro} and E^{ms} was approved for commercial use in Germany (Agency).

1.3.2 Immune Responses Elicited by the BVDV Vaccines

While both MLVs and KVs vaccines confer protection, their efficacy and the type of immune responses they induce are quite different (Griebel, 2015; Reber et al., 2006). MLVs stimulate the production of neutralizing antibodies and BVDV-specific CD4⁺ and CD8⁺ T cells (Bittar et al., 2020; Endsley, Quade, Terhaar, & Roth, 2002; Zimmerman et al., 2006). On the other hand, KVs require higher antigen concentrations combined with strong adjuvants to generate neutralizing antibody responses and CD4⁺ T cell activation (M. Beer et al., 2000; Platt, Coutu, Meinert, & Roth, 2008; Walz et al., 2018). Immune responses from MLVs target structural and non-structural BVDV proteins, whereas KVs responses are directed to structural antigens (Griebel, 2015). Notably, there has been no evidence of induction of BVDV-specific cytotoxic CD8⁺ T lymphocyte (CTL) responses by either MLVs or KVs. One crucial role of BVDV vaccination is in preventing the birth of PI calves in breeding cattle, given that PI animals are BVDV reservoirs that shed the virus (R.

W. Fulton, 2013; Walz et al., 2018; Xue, Mattick, & Smith, 2011). MLV vaccines are generally more effective in protecting fetuses in pregnant cows than KVs (Fairbanks, Rinehart, Ohnesorge, Loughin, & Chase, 2004; Walz et al., 2018; Xue et al., 2011; Zimmer et al., 2002). However, there are serious risks associated with the administration of MLVs in pregnant cows (Volker Moennig & Liess, 1995).

Calves normally elicit high cross-neutralizing antibody titers (1:256 to 1:1024) when exposed to homologous BVDV strains within the same sub-genotype. However, strains from heterologous sub-genotypes tend to elicit sub-optimal to low cross-neutralizing titers (1:4 to 1:128) (R. W. Fulton et al., 1997; Klimowicz-Bodys et al., 2021). This indicates that BVDV strains contain both unique and shared neutralizing epitopes (Botton, Da-Silva, Brum, Weiblen, & Flores, 1998). Due to this antigenic diversity, many commercial BVDV vaccines available in the United States are designed to be bivalent (including BVDV-1a and -2a strains) or trivalent (including BVDV-1a, -1b, and -2a strains) (Walz et al., 2020). These multivalent vaccines exhibit increased neutralizing antibody titers against homologous BVDV strains (R. W. Fulton et al., 1997; Sozzi et al., 2020), and heterologous strains, depending on the strain composition of the commercial vaccine, which is sometimes protective as measured by lower clinical sign indices (Robert W Fulton et al., 2020; Xue et al., 2011; Xue, Mattick, Smith, Umbaugh, & Trigo, 2010).

1.3.3 Limitations Associated with the Current BVDV Vaccines

Due to antigenic variability, there is a widening gap between current commercial vaccine strains and efficacy against heterologous strains (Robert W Fulton et al., 2020; Klimowicz-Bodys et al., 2022; B. W. Newcomer et al., 2017). Nearly all MLVs or KVs are currently formulated with a mixture of one representative BVDV-1a and -2a strains (Robert W Fulton et al., 2020; Melkamsew,

Tessema, & Paeshuyse, 2025), which may have led to BVDV-1b emerging as the most prevalent sub-genotype in the United States (Robert W Fulton et al., 2006; Julia F Ridpath, Fulton, Kirkland, & Neill, 2010; Julia F. Ridpath et al., 2011; Stronga, 2013). Typically, vaccines elicit neutralizing antibodies against heterologous strains, which may or may not always confer protection. Several examples from feedlot cattle showing that MLVs or KVs were not effective at conferring cross-protection have been documented. In the United States, additional genotypes 2b and 2c were recently identified, which contained genetic and antigenic differences from BVDV-2a (John D Neill et al., 2019). Notably, sera from cattle immunized with several BVDV-2a MLV vaccines varied in their ability to neutralize BVDV-2c strains. Vaccine strains induced protective neutralization titers against some, but not all, BVDV-1a, -1b, and -2a strains and significantly low neutralizing titers against BVDV-2c strains (Robert W Fulton et al., 2020). A similar situation was observed in sheep from earlier studies, where genotype-specific MLV vaccines were ineffective at conferring cross-protection against diverse BVDV strains (Botton et al., 1998). In another study, a MLV Mucosiffa vaccine containing the BVDV-1a vaccine strain induced neutralization titers against BVDV-1a that were similar to those of the BVDV-1b strain, but lower titers were observed against circulating European field strains of BVDV-1f and -1e, and significantly lower titers against BVDV-2a at 28 days after vaccination, that remained low for the entire 363-day study period (Grange, Mindeguia, Gisbert, & Meyer, 2023). Additionally, a commercial polyvalent MLV elicited mean neutralization titers above the protective 1:256 titer against field strains of BVDV-1a, -1d, -1f, -1h, -1i, but titers lower than the protective threshold were observed against BVDV-1b (1:66.7), -2b (1:45.2), and 1r field strains in Turkish cattle (Alpay & Yeşilbaş, 2019). Similarly, in Brazil, BVDV-1 and BVDV-2 inactivated vaccines induced neutralizing antibodies against some but not all BVDV-1 (NADL, Singer) field strains, while no neutralizing antibodies were induced

against the BVDV-2 (VS253) strain (Baccili et al., 2019). This antigenic variation is measured by both humoral viral neutralizing (VN) titers and cell-mediated immune responses. BVDV-1a and -2a specific MLVs induce higher IFN- γ mRNA expression by CD4⁺, CD8⁺, and CD335⁺ T cells in PBMCs stimulated with homologous, but not heterologous sub-genotype strain (Falkenberg et al., 2021). Therefore, current MLV vaccines do not provide broad epitope coverage of the circulating diverse BVDV strains and, thus, are poor in conferring cross-protection.

Immunizing cattle intranasally may be more rational, as it prevents infection at the site of pathogen entry (Lemon & McMenamy, 2021). Intranasal immunization primes localized protective immune responses by activating the Nasal-associated lymphoid tissues. Importantly, intranasal immunization activates immune responses even in the presence of maternally derived antibodies (Lemon & McMenamy, 2021). However, most current MLV and KV vaccines are administered parenterally (Falkenberg, Dassanayake, Crawford, Sarlo Davila, & Boggiatto, 2023; Grange et al., 2023; Masic et al., 2021; Melkamsew et al., 2025; Taberner et al., 2024). Additionally, in calves younger than six months, the efficacy of parenteral vaccines can be significantly reduced due to interference from maternal antibodies and the potential for complement-mediated clearance (J. Ellis, K. West, V. Cortese, C. Konoby, and D. Weigel., 2001; R. W. Fulton, J. F. Ridpath, S. Ore, A. W. Confer, J. T. Saliki, L. J. Burge, and M. E. Payton., 2005.; Howard, Clarke, Sopp, & Brownlie, 1992; Zimmerman et al., 2006). As a result, immunization with BVDV-1 and -2 vaccines is delayed for about six months to allow maternal antibodies to wane. Unfortunately, the presence of PI calves in the herd increases the risk of infection, especially as protective maternal antibody titers may wane earlier than six months (Brar, 1978.; Robert W. Fulton et al., 2004). Furthermore, in the presence of PI calves, the lack of effective testing methods

at the chute side to identify these animals poses a great risk to unvaccinated cattle (Robert W Fulton et al., 2006).

Several safety concerns have also been linked to BVDV MLV and KV vaccines (J. F. Ridpath, 2013). Possible contamination with adventitious wild-type virus can lead to unintended infection and spread of the virus within the cattle herd (Palomares, Marley, Givens, Gallardo, & Brock, 2013). Additionally, MLV vaccines, similar to the wild-type BVDV, may cause immunosuppression and lead to post-vaccination mucosal disease (Chase, Hurley, & Reber, 2008). MLVs can also infect the reproductive organs, with BVDV antigens being detectable in ovaries for up to 30 days after vaccination, raising concerns about decreased fertility in vaccinated heifers (R.W. Fulton et al., 2003; Grooms, Brock, & Ward, 1998). Furthermore, MLV vaccines can lead to *in utero* infections, and the virus can be shed by vaccinated animals, which may result in environmental contamination (Kelling, 2004; van Oirschot, 1999).

Despite having access to licensed vaccines, some countries opt for biosecurity control measures instead of vaccination, as BVDV vaccines are not 100% effective (V. Moennig, Houe, & Lindberg, 2005; Scharnbock et al., 2018; Ståhl & Alenius, 2012). A number of countries in Europe, such as Norway, Sweden, Austria, Finland, and Denmark prohibit the use of BVDV vaccines (V. Moennig et al., 2005; Scharnbock et al., 2018).

1.3.4 Protective BVDV Antigens

BVDV infection or vaccination elicits antibodies against BVDV E^{rns}, E1, E2, and NS2-3 antigens (Steven R. Bolin, 1993). However, only the E2 antigen contains protective neutralizing epitopes (Deregt, van Rijn, Wiens, & van den Hurk, 1998). Cell mediated immunity is crucial for the clearance of BVD since depletion of CD4⁺ T cells in cattle has been shown to delay the resolution

of acute BVDV infection (Howard et al., 1992). CD4⁺ T cell responses, elicited by the N^{pro}, Capsid, E^{rns}, E2, and NS2-3 antigens, contribute to protection against BVDV infection (T. Collen, Carr, Parsons, Charleston, & Morrison, 2002). Conserved regions of the E2 and NS2-3 antigens also contain MHC *DR*-restricted T cell epitopes (M. Beer, G. Wolf, J. Pichler, A. Wolfmeyer, and O. R. Kaaden., 1997.; T. Collen, and W.I. Morrison., 2000.; Deregt et al., 2005; Hegde, 1997; Howard et al., 1992). It has also been shown that in addition to E2 and NS2-3, the NS4-5 antigens contain CD4⁺ T-cell epitopes that induce strong IFN- γ secretion (139, 143) and novel potent IFN- γ -inducing CD8⁺ T cell epitopes that are highly conserved among diverse BVDV strains (Sangewar et al., 2021). Certain regions of the E2, NS2-3, and NS4-5 antigens are conserved among BVDV strains (Deng, 1992). However, these antigens, particularly E2, also contain variable regions within antigen domains that are crucial for eliciting protective immunity, thus contributing to the variable cross-reactivity observed among BVDV strains, which, in part, explains why current vaccines are poor at conferring cross-protection against diverse BVDV strains (Botton et al., 1998). A vaccine containing all defined protective B and T cell epitopes from diverse BVDV-1 and -2 strains is yet to be developed and tested in calves. Numerous vaccine candidates have been developed and tested in cattle, including DNA-based vaccines, recombinant BVDV subunit vaccines, live viral vectored vaccines, virus-like particles and replicons, chimeric BVDV vaccines, and epitope-based vaccines.

1.3.5 Nucleic Acid-based BVDV Vaccines

It was previously shown that a DNA plasmid encoding BVDV-1a E2 antigen conferred partial protection against BVDV-1b strain in calves (Harpin, Hurley, Mbikay, Talbot, & Elazhary, 1999; Nobiron, 2003). While DNA vaccines elicit strong immune responses in small animals such as mice and guinea pigs, they are normally less potent in larger animals. Different strategies have

been explored to enhance their potency, including electroporation-based delivery or co-administration with a DNA plasmid encoding interleukin-2 (IL-2) or granulocyte-macrophage colony-stimulating factor (GM-CSF), which resulted in reduced viremia in vaccinated cattle following BVDV challenge (Nobiron, Thompson, Brownlie, & Collins, 2001; van Drunen Littel-van den Hurk et al., 2010). Priming the calves with DNA plasmids encoding E2 and subsequently boosting with recombinant E2 antigen conferred complete protection (Liang et al., 2008). Furthermore, a DNA plasmid expressing a Retinoic acid-inducible gene I (RIG-I) agonist, along with a BVDV-1a antigen, E2 or NS3, were evaluated in calves. A combination of the E2- and NS3-encoding plasmids elicited cross-neutralizing antibodies against BVDV-1a strains (El-Attar LM, Thomas, Luke, J, & Brownlie, 2015). Recently, a plasmid DNA expressing the E2 antigen inserted on the hinge region of the mouse lysosome-associated membrane protein 1 (LAMP1) as an adjuvant was observed to increase neutralizing antibody titers and E2-specific IFN- γ production compared to the non-adjuvanted E2 formulation in cattle (Sakai et al., 2024). Advancements in molecular vaccine technology are now a popular platform due to high scalability (M. A. Liu, 2019). Therefore, developing highly effective DNA- or RNA-based BVDV vaccines is achievable.

The successful use of mRNA vaccine for SARS-Cov2 has changed the vaccine development landscape for many viral infectious diseases. In the 1990s, CTL activity against influenza had been induced by immunizing mice with influenza nucleoprotein mRNA encapsulated in liposomes, indicating that highly effective mRNA vaccines could be developed quickly and mass-produced cheaply than some of the conventional vaccines (Beissert et al., 2020; Martinon et al., 1993; Pardi, Hogan, Porter, & Weissman, 2018; Sharif, Alzahrani, Ahmed, & Dey, 2021). The mRNA vaccine provides an assurance of rapid progress in development of vaccines to control many diseases (Chaudhary, Weissman, & Whitehead, 2021). *In vitro* transcribed synthetic

mRNA transiently expresses a protein similar to that produced by its native mRNA. Endosomal and cytosolic innate immune receptors and certain cell surface pattern recognition receptors recognize exogenous mRNA, making them naturally immunostimulatory. However, they can be rapidly degraded, thus requiring protection and a delivery vehicle such as lipid nanoparticles to enable delivery into the cell cytosol for expression of the encoded antigen and thereby induce immune responses (Chaudhary et al., 2021; N. Chen et al., 2017; N. N. Zhang et al., 2020). In the case of BVDV and other Pestiviruses, E2-based mRNA vaccines can elicit efficacious humoral and cellular mediated immune responses (Le, Sun, Chang, Zhang, & Yin, 2022).

1.3.6 Recombinant BVDV Subunit Vaccines

Subunit vaccines are considered the safest approach for developing novel vaccines (Moyle & Toth, 2013). When formulated with an adjuvant, recombinant E2 antigen elicits high neutralizing antibody titers against cognate BVDV strains (Pecora et al., 2012; R. L. Toth, Nettleton, & McCrae, 1999). Different expression platforms can be used to produce the soluble domain of the E2 antigen efficiently (Asfor, Wakeley, Drew, & Paton, 2014; Bhuyan et al., 2018; R. L. Toth et al., 1999). It has been shown that although monovalent E2-based subunit vaccines induce BVDV-neutralizing antibodies, the level of protection conferred significantly varies depending on the expression platform used (Chimeno Zoth, Leunda, Odeón, & Taboga, 2007; Pecora et al., 2012). Expressing E2 antigens using recombinant baculovirus elicits neutralizing antibodies in the host, but it does not confer complete protection (Chimeno Zoth et al., 2007). Conversely, expressing E2 antigen in mammalian cells (including HEK293, CHO, or MDBK cells) confers protection using a dose as low as 5 µg per calf (S. R. Bolin & Ridpath, 1996; Pecora et al., 2012; Sadat, Snider, Garg, Brownlie, & van Drunen Littel-van den Hurk, 2017; Thomas, Young, Heaney, Collins, &

Brownlie, 2009). The superior efficacy of the mammalian cell-expressed E2 subunit vaccines is likely due to differences in post-translational modifications between the two expression systems (Thomas et al., 2009). Plants can also be used to express protein. A truncated E2 expressed in tobacco plant induced specific antibodies upon inoculation of guinea pigs (Nelson, Marconi, Periolo, La Torre, & Alvarez, 2012). Intramuscular immunization of cattle with 3 µg of a purified chimeric E2 protein, fused to a single-chain for targeting to MHC molecule, expressed in alfalfa plant induced strong antibody response, which was protective upon challenge (Aguirreburualde et al., 2013).

Neutralizing antibody response, along with cytotoxic T Lymphocyte activity that destroys BVDV-infected cells, contributes to resolving acute BVDV infection (Chase, 2013). A recombinant BVDV-2 E2 antigen, expressed in mammalian cells, was formulated with an adjuvant containing a toll-like receptor (TLR) 3 agonist and a Th1/Th2 response enhancer, elicited BVDV-neutralizing antibodies along with E2-specific CD4⁺ and CD8⁺ T cell responses (Snider, Garg, Brownlie, van den Hurk, & Hurk, 2014). The elicited Th1 and Th2 immune responses prevented viremia in vaccinated animals post-challenge with a virulent BVDV-2 strain (Sadat et al., 2017). Similarly, a biomimetic particle-based vaccine was developed as a prototype BVDV vaccine containing encapsulated NS3 antigen, a TLR 3 agonist, and E2 antigen displayed on the particle surface. This prototype vaccine conferred protection comparable to a commercial KV vaccine (Riitho et al., 2017). In a mouse model, animals immunized with the MF59–and CPG-ODN–adjuvanted recombinant E^{rms}-E2 protein expressed in *E. coli* had higher levels of IL-4 and BVDV-specific antibody titers than mice immunized with individual E2 and E^{rms} formulations. Furthermore, the E2-E^{rms} immunized mice had greater proportions of CD4⁺IFN-γ⁺, CD8⁺IFN-γ⁺ T cells, and CD3⁺TNF-α⁺ T cells, which translated to a better protective efficacy on challenge (S.

Wang et al., 2020). A subunit vaccine containing purified recombinant E2 and NS2-3 mosaic antigens that incorporate determinants that are conserved among BVDV-1a, b, and BVDV-2 genotypes, conferred protection against BVDV-1 & 2 strains (Sangewar et al., 2020). E^{ms} conjugated to LTB (a subunit of the heat-labile enterotoxin of *Escherichia coli*) in M59 adjuvant provided enhanced protection to mice following challenge with 2 BVDV strains, primarily because of induced T cell immunity, suggesting potential for development of broadly protective vaccines (Nashar, Williams, & Hirst, 1998; S.-H. Wang et al., 2021).

A chimeric E2 antigen, containing E2 antigens from BVDV-1a, -1b, and -2a strains, was designed to target MHC II-expressing antigen-presenting cells, eliciting high neutralizing antibody titers against all three cognate strains (D. Bellido et al., 2020; Pecora et al., 2015). Upon challenge with heterologous BVDV-1b strain or a BVDV-2b strain (n=2), the multivalent vaccine conferred complete protection against BVDV-1b and partial protection against BVDV-2b (Pecora et al., 2015). In 2017, this recombinant vaccine was the first licensed BVDV subunit vaccine approved for field application in Argentina (D. Bellido et al., 2020). It is produced at a large scale in insect cells, has enhanced immunogenicity, and was 100% protective against one of the two tested BVDV strains (D. Bellido et al., 2020; Pecora et al., 2015). In subsequent studies in which 2,992 feedlot cattle were used, this vaccine induced E2-specific neutralizing antibodies that reduced the levels of the BVDV virus circulation in the treated group, leading to fewer cases needing BRD treatment (Demian Bellido et al., 2024). However, its protective efficacy against more diverse BVDV strains in more animals remains to be determined.

In a mouse model, intraperitoneal immunization with mammalian cells generated from recombinant BVDV-1a NADL E2 antigen formulated with Montanide ISA 61 VG adjuvant, elicited BVDV-specific neutralizing antibodies, with VN titers of 1:8192 and 1:16,384 at seven

and twenty-one days post-challenge, respectively, and significantly reduced histopathologic lesions in the lungs, liver, and brain, providing better protective efficacy following challenge (Gómez-Romero et al., 2023). In another mouse model, recombinant NS5A, E0 (E^{ms}), E2, and mE2T (a modified truncated version of E2) antigens expressed in prokaryotes and emulsified with Freund's adjuvant elicited BVDV-specific neutralizing antibodies, with VN titers of 1:18.2, and induced BVDV-specific splenic T cell proliferation, providing better protective efficacy (Y.-H. Li et al., 2022).

A secreted form of E2 glycoprotein, in which the transmembrane anchor domain at the C-terminal is truncated and expressed in BHK21 cells, when used to immunize mice intraperitoneally without adjuvant, induced production of BVDV-specific total IgG, IgG₁ and IgG_{2a}, stimulated lymphocyte proliferation, increased production of splenic CD4⁺ and CD8⁺ T cells, and increased production of IL-12, IL-4 and IFN- γ cytokines in sera (Lo, Ryan, Luke, Chang, & Wu, 2023). Intranasal administration of HEK293A-expressed E2 antigen fused with Bovine IgG1 Fc (E2-Fc), but not a cognate intramuscularly delivered E2 antigen fused with ferritin (E2ft), primed potent BVDV-specific mucosal and serum IgA and SIgA, with VN titers of up to 1:64 in calves, induced more IFN- γ -secreting CD4⁺ and CD8⁺ T cells, translating to better protective efficacy (Cheng et al., 2023). Recombinant BVDV-NADL E^{ms} antigen expressed in CHO cells was shown to be immunogenic in New Zealand white rabbits, elicited production of BVDV-specific antibodies that were detected by seven days post-prime and had viral neutralization titers of up to 2.71 log₁₀ in rabbits (Y. Li et al., 2023). Another E2 subunit vaccine derived from five BVDV sub-genotypes; 1a, 1b, 1c, 1d, 1e, expressed in CHO cells and formulated in Montanide ISA 61 VG and intramuscularly immunized sheep and cattle, elicited protective neutralizing antibodies against

BVDV-1a similar to those of a commercial BVDV-1a MLV vaccine, and significantly increased transcription of BVDV specific IFN- γ and IL-12 cytokines (Avello et al., 2024).

A novel BVDV subunit vaccine consisting of live attenuated BRSV, genetically modified BoHV-1, inactivated PI3, and BVDV-1 and -2 E2 recombinant antigen, designated as DIVENCE®, was recently developed and tested in Spain. Intramuscular immunization of heifers before breeding significantly increased neutralizing antibody titers from 42 days post-vaccination, and protected 94% of fetuses from BVDV-1 and -2 upon challenge, as judged by reduced rectal temperature, leukopenia, viral shedding, and viremia in blood (Taberner et al., 2024). Immunization of calves in commercial fattening units resulted in lower morbidity, lower anti-microbial treatments and higher weight gains, showing an improved performance crucial for economic outcomes (Tapiolas et al., 2024). In an immunogenicity study, the subunit vaccine elicited neutralizing antibodies to BVDV-Singer and BVDV-2 VV-670 that persisted until 591 days. Importantly, neutralizing antibodies were detected in milk from vaccinated animals (Montbrau et al., 2024).

1.3.7 Live virus-vectored BVDV Vaccines

Recombinant viral vectors expressing BVDV antigens elicit potent BVDV-specific cellular and humoral immune responses following vaccination (Lokhandwala et al., 2017; Rosas et al., 2007). One study demonstrated that a recombinant equine herpesvirus-1 (EHV-1) encoding the BVDV-1b Capsid, E^{rns}, E1, and E2 proteins elicited neutralizing antibodies that resulted in reduced viremia in peripheral blood and viral shedding in nasal secretions following BVDV-1b challenge (Rosas et al., 2007). Similarly, intranasal vaccination of cattle with a recombinant bovine herpesvirus-4 (BoHV-4) expressing BVDV E2 antigen elicited BVDV-neutralizing antibodies (Williams et al., 2019). A replicon derived from the Venezuelan Equine Encephalitis (VEE) virus evaluated in cattle

as a delivery system for the BVDV-1b E2 antigens conferred some degree of protection against homologous BVDV-1b challenge (Loy et al., 2013). Delivery of BVDV-1b E2 antigen using the EHV-1 and the alphavirus-based replicon vectors also elicited cross-neutralizing antibody responses against BVDV-2. However, the BVDV-specific T cell responses elicited by these vaccines were not assessed. As previously observed for Hepatitis C Virus (HCV) mosaic vaccine prototype, immunizing calves with a cocktail of recombinant adenoviruses expressing mosaic N^{pro}, E2, and NS2-3 antigens from BVDV-1 and -2 strains elicits broadly neutralizing BVDV-1 and -2 antibodies and high levels of protective BVDV-specific CD4⁺ T cell responses (Lokhandwala et al., 2017; Yusim et al., 2013). BVDV E2 gene inserted in replication-defective adenovirus and controlled by three different promoters produced high levels of neutralizing antibodies in mice. T_H cell proliferation with increased IFN- γ levels was observed in the vaccinated mice (Elahi et al., 1999). Although the antigenic structure of E2 in BVDV-1 and BVDV-2 is conserved, type-specific epitope differences necessitate a mosaic vaccine (Cui et al., 2016; Jelsma, Loeffen, van Beuningen, & van Rijn, 2013). The adenoviral vectored BVDV mosaic antigens outperformed the commercial MLV, completely protecting vaccinated calves against virulent BVDV-2 challenge (Lokhandwala et al., 2017). These candidate BVDV vaccines, generated using replicating or non-replicating viral vectors to induce robust cross-protective immunity, present a promising alternative to MLVs for protecting herds against the diverse BVDV strains. A chimeric CSFV containing BVDV E2 as a marker has been approved for commercial use after its safety and efficacy was confirmed experimentally (Blome et al., 2014). Balb/C mice orally administered with a genetically engineered *Lactobacillus* vaccine expressing BVDV E2 fused with cholera toxin B subunit as adjuvant were protected against challenge with BVDV strain ZD-2018 (Jia et al., 2020). Another *Lactobacillus* expressing BVDV E2 fused with a dendritic cell targeting peptide as an oral

probiotic vaccine induced efficient mucosal, humoral, and cellular immune responses that provided protection upon challenge (Y. Wang et al., 2019).

1.3.8 Virus Like Particles and Replicons

Virus-like particles (VLPs) offer a promising approach as antigen delivery platforms. Their small size makes them suitable for uptake by DCs, which process the antigen and present peptides to T cells in the context of MHC class I and II molecules. The mechanisms by which VLPs activate APCs have been described; these include processing of VLP particles inside the endocytic vesicles of APCs without reaching the cytosol and the peptides derived from these VLPs bind pre-existing MHC class I molecules as has been described for HbsAg (Hepatitis B virus small envelope protein) (Sedlik, Saron, Saraseca, Casal, & Leclerc, 1997). Hepatitis C can be used as a model for a generation of other Flavivirus VLPs, including BVDV. Insect cell produced recombinant hepatitis C virus (HCV)-like particles having the structural proteins core, E1, and E2 resemble the HCV virions and induce strong and broad B and T cell responses in mice and baboons (Elmowalid et al., 2007; Torresi, Johnson, & Wedemeyer, 2011). Similarly, particles that mimic wild-type BVDV induced antibody and T cell responses in cattle. However, upon challenge, the induced immune responses did not control viremia as well as the KV vaccine, perhaps because the VLPs containing BVDV antigens were produced in non-mammalian systems (Riitho et al., 2017). VLPs containing E2 and E^{ms} dimer formulated with ISA201 adjuvant induced neutralizing antibodies, enhanced T cell proliferation as well as secretion of Th1-associated IFN- γ and Th2-associated IL-4 cytokines. In addition, increased levels of CD3⁺CD4⁺T cells and CD3⁺CD8⁺T cells were detected in vaccinated mice (Z. Wang et al., 2021). The strong immune response was comparable to that induced by a killed commercial vaccine, supporting use of VLPs as a vaccine. Maternally

transferred immunoglobulins following immunization with Semliki Forest virus replicon expressing the E2 glycoprotein of Classical Swine Fever Virus (CSFV), a pestivirus, protected piglets upon challenge with CSFV (Sedlik et al., 1997). VLPs can be adapted to be successfully expressed in various systems. For example, C-E^{rns}-E1-E2 region of BVDV-1 was cloned into a pFastBacDaul vector for producing VLPs in Sf9 insect cells, which were mixed with Montanide ISA-201 adjuvant prior to inoculating guinea pigs. Following challenge, the vaccinated guinea pigs had lower viral load compared to the controls (Gao et al., 2022).

Von Magnus first described the defective interfering (DI) particles for the influenza virus, which result naturally from degenerate forms of the genome, defective viral genomes (DVG), created by point mutations, frame shift, deletions, insertions or rearrangements or all (Magnus, 1947). DVGs cannot self-replicate unless helped and packaged by complementation with a wild-type virus. The genetics of DVGs, which are found in all virus systems, has allowed reverse engineering of DVGs that results in replicons that may be used for developing immunizing agents. Self-amplifying recombinant RNA or replicons do not produce infectious virions, but express proteins adequate for their own replication. Since replicons persist in the cells, they are passed to individual cells during division (Khromykh, 2000; Lundstrom, 2005). Three CSFV-E2 deletion mutants that were unable to produce viable viruses, were rescued by trans-complementation in SK6 cell lines constitutively expressing E2. The rescued viruses grew in SK6 cells following primary infection and expressed viral proteins but were not transmissible. Intradermal inoculation of pigs with E2-complemented CSF virus replicon particles (VRP) elicited a protective immune response (Van Gennip, Bouma, Van Rijn, Widjojoatmodjo, & Moormann, 2002). A replication-competent mutant Classical Swine Fever Virus (CSFV) strain Alfort/187, created by deleting the sequence encoding the 227 amino acids of the glycoprotein E^{rns} (A187delE^{rns}), was infectious in

cell culture and produced VRPs without yielding infectious progeny virus. Intradermal immunization with the VRPs protected pigs challenged with a highly virulent CSFV strain (Reimann et al., 2010). *In vitro* tests indicated elicitation of an efficacious neutralizing antibody response and an increase in IFN- γ producing cells in vaccinated pigs (Frey et al., 2006; Moser, Stettler, Tratschin, & Hofmann, 1999). A transcomplemented set of BVDV deletion mutant replicons replicated without infectious progeny, and inoculation of the pseudoviruses into calves induced specific antibodies, and the animals were protected against challenge (Reimann, Semmler, & Beer, 2007). Recently, a red-spotted grouper nervous necrosis virus (RGNNV) VLPs presenting the BVDV E2 protein, was observed to induce robust antibodies in mice, which neutralized strongly (1:800 to 1:3,200) or weakly ($\sim$ 1:10) homologous or heterologous BVDV strains, respectively (Katsura et al., 2023).

Virosomes are composed of relevant protein(s) or glycoprotein(s) of a virus embedded in a lipid bi-layer membrane structure that allows the fusion with the target cells, for example, dendritic cell, to generate an effective humoral and/or cell-mediated immune response on inoculation. Virosomes were first generated using liposomes, hemagglutinin, and neuraminidase to derive the protruding spike protein of the influenza virus in the membrane vesicles. Fusion-active virosomes mimic viral envelopes for binding and fusion with the target cells. Following attachment and/or fusion with APCs, antigens are delivered onto the surface or into the cytosol to induce an effective T_H1 and T_H2 cell response (Almeida, Edwards, Brand, & Heath, 1975; Arkema, Huckriede, Schoen, Wilschut, & Daemen, 2000; Huckriede et al., 2005).

1.3.9 Chimeric BVDV Vaccines

Given that BVDV is a part of the BRD complex, developing a chimeric vaccine against multiple bovine viral pathogens would be an optimal strategy for reducing BRD in cattle herds (Williams et al., 2019). Chimeric prototype vaccines containing BVDV and BoHV-1 have been developed to confer dual protection against these two co-circulating immunosuppressive pathogens (Chowdhury et al., 2021; Williams et al., 2019). When evaluated in cattle, a chimeric BoHV-1 vectored BVDV-2 E2 vaccine elicited BVDV-2 and BoHV-1 cross-neutralizing antibodies along with BVDV-1 and -2 cross-reactive T cells that were recalled upon BVDV-2 challenge (Chowdhury et al., 2021). Similarities in gene organization and sequences allow for interchanging the genes/parts of genes between pestiviruses. A dual vaccine was developed by inserting the E1 and E2 antigens from a BVDV-2 strain into the backbone of a BVDV-1b strain. This backbone had an attenuating deletion within the N^{pro} gene, and a substituted E^{ms} from Bungowannah virus, which is a heterologous *Pestivirus* (BuPv, species *Pestivirus F*), for differentiation of infected from vaccinated animals (DIVA) (Koethe et al., 2020). The chimeric dual vaccine, either as a single dose live vaccine or double dose of inactivated vaccine, was effective against BVDV-1 and -2 infection in the vaccinated calves (Koethe et al., 2020; Koethe et al., 2022). The successful development of DIVA compatible chimeric vaccines needs to be assessed for their efficacy and safety before they can be considered for field application (Koethe et al., 2022; Luo et al., 2012).

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nucleases (CRISPR/Cas) system has proven to be a potent procedure for DNA editing (Hsu, Lander, & Zhang, 2014). The Cas9 system has been used to engineer viruses to study gene functions, vector and vaccine development (Bi et al., 2014; Hübner, Keil, Kabuuka, Mettenleiter, & Fuchs, 2018; Puschnik, Majzoub, Ooi, & Carette, 2017; Teng, Yao, Nair, & Luo, 2021; Vilela,

Rohaim, & Munir, 2020; Yuan et al., 2015; Y. Zhang et al., 2018). CRISPR/Cas9 procedure was used to knock out, simultaneously, the gE gene or gE and US9 genes of a respiratory disease-causing virus, Bovine herpesvirus type I (BoHV-1), for its use as a deletion mutant vaccine and as a delivery vector for other viral genes (C.-Y. Liu et al., 2022). This technique would provide a rapid system to improve on a reported BoHV1- BVDV vaccine. A multigene deleted BoHV-1 (BoHV-1 QMV) vector carrying BVDV-2 E2 and E^{ms}-bovine granulocyte-macrophage colony-stimulating factor (E^{ms}-GM-CSF), induced BVDV-2 neutralizing antibodies and additionally, cross-reactive cellular immune responses to BVDV-1 and BVDV-2. Following challenge, the clinicopathology outcome of the vaccinated calves was better than the calves vaccinated with a commercial vaccine (Chowdhury et al., 2021).

Newer multimeric protein scaffold particles (MSRP) and multiepitope peptides are platforms that have been utilized to develop vaccines against viral infections such as Rift Valley Fever and Middle East Respiratory Syndrome. Bioinformatics allows the use of contemporary computational techniques, *in silico*, to select and/or design molecules that are potentially immunoreactive to induce a protective response (CTL, T_H, and B cells) after inoculation of the synthesized antigen into hosts (G. Liu et al., 2020; Palatnik-de-Sousa, Soares, & Rosa, 2018; L. Zhang, 2018). Furthermore, structural maps of identified epitopes can be visualized, and for B cell epitopes, interactions with the specific antibody can be shown at the atomic level using X-ray crystallography (Saadi, Karkhah, & Nouri, 2017). Alternatively, peptide libraries are screened to identify B cell or T cell epitopes, which are further evaluated and analyzed for confirming the reactivity using *in vitro* techniques (Batoool et al., 2018; Rogovskyy et al., 2019; Sangewar et al., 2021). The identified molecules, which can be broadly reactive, are then either used as chemical conjugates or expressed by a vector following insertion of a synthetic gene (Aguilar-Noriega et

al., 2014). Putative epitopes present in BVDV E2 antigen that may potentially elicit neutralizing antibodies were mapped *in silico* as potential targets for vaccine development (Al-Kubati, Hussien, Kandeel, Al-Mubarak, & Hemida, 2021). Screening of a 12-mer peptide library with an anti-BVDV serum led to the identification of a mimotope that further led to identification of a putative B cell epitope and inoculation of mice with a cognate synthetic peptide elicited antibodies that were reactive to BVDV, but did not neutralize viral infectivity (X. Chen, Ding, Zhu, & Zhang, 2021).

1.3.10 Adjuvants

Most good vaccines are efficacious because use of an effective adjuvant can safely potentiate humoral and cell-mediated immune responses to confer protection against a pathogen (Altenburg, Rimmelzwaan, & de Vries, 2015; Reed, Orr, & Fox, 2013). The selection of an adjuvant for use is empirically determined based on the pathogen and route of administration. It is clear that CD8⁺ T cells are essential in clearing viral infections, thus induction of virus specific CD8⁺ T cells responses make a vaccine more efficacious (Rapaka, Cross, & McArthur, 2021; Schmidt & Varga, 2018; Seder, Darrah, & Roederer, 2008). Formulations of some adjuvants include alum, water-in-oil, oil-in-water, saponins, polymers such as chitosan, dendrimers, nanoparticles, Toll-like receptor agonist, cytokines (for example IL-2) and antibody targeting receptors (for example anti-CD40; anti-MHC) (Arya, Lin, Zhou, Gao, & Huang, 2020; Havenar-Daughton et al., 2019; Keler, He, Ramakrishna, & Champion, 2007; Stils, 2005). Calves inoculated with three experimental killed BVDV vaccines in an adjuvant composed of Quil-A, cholesterol and dimethyldioctadecylammonium (DDA) bromide had better immune response than an MLV vaccine (Julia F. Ridpath et al., 2010). Use of carbomer to stabilize a saponin adjuvanted killed

BVDV vaccine increased the neutralizing antibodies that lasted longer in calves compared to calves vaccinated with a commercial vaccine that used lactoalbumin as the stabilizer (El Fadeel et al., 2021). When co-injected, IL-2 and granulocyte-macrophage stimulating factor (GM-CSF) plasmid constructs increased both the humoral and cellular responses elicited in mice immunized with a BVDV E2 plasmid DNA construct (Nobiron et al., 2001). An anti-swine CD40 monoclonal antibody, has been shown to upregulate MHC II expression in peripheral blood monocytes (PBMCs) and stimulate IL1, IL6, IL-8, and TNF expression by PBMCs (Martin et al., 2017). Viral antigens can be adjuvanted with Quil-A in the form of Immunostimulating complexes (ISCOMs), which protect antigens by forming a cage-like structure by hydrophobic interactions (Paillot, Grimmett, Elton, & Daly, 2008). When inoculated in calves, BVDV antigen formulated with ISCOMs induced high levels of neutralization antibodies against diverse BVDV isolates (Kamstrup, Roensholt, Jensen, & Dalsgaard, 1999). A codon optimized E2 gene expressing recombinant BVDV E2 protein delivered in silica vessels with Quil-A for controlled release induced ten times higher antibody levels and higher cell-mediated responses than the E2 in Quil A alone following inoculation in mice. The release rate of the antigen within the silica vessels can be regulated by the size of the pores in the silica (Mody et al., 2014). Calves vaccinated with recombinant BVDV-2 E2 formulated in a novel adjuvant composed of poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP), a toll-like receptor (TLR) agonist, and an innate defense regulator (IDR) peptide plus either CpG ODN or poly(I:C) had no clinical disease and remained free of viremia post challenge with a high dose of BVDV-2. This contrasts the results obtained when E2 protein formulated in oil-in-water plus cpG ODN was used for immunization (Liang, van den Hurk, Babiuk, & van Drunen Littel-van den Hurk, 2006; Snider et al., 2014). Recently, melatonin inclusion in an MF59 adjuvanted BVDV E^{rns}-LTB was suggested as an

immunopotentiator by inhibiting the κ B signaling pathway that induces inflammation following BVDV infection (Y.-X. Wang, Yang, Zhang, Wang, & Wang, 2021). Peptides, when used as a vaccine, need to be modified to evoke an immune response on administration. Strong humoral and cellular responses were induced in mice following immunization with OVA peptides conjugated to polystyrene nanoparticles (Fifis, Mottram, Bogdanoska, Hanley, & Plebanski, 2004). Multiple FMD virus peptides conjugated individually or as a mixture to nanoparticles, induced significant cell mediated immune response as well as humoral response (Greenwood et al., 2008). In a proof-of-concept study, similar results were obtained using BVDV E2 conjugated to silica nanoparticles, except that the antibody levels were slightly lower than those obtained by immunization of sheep with E2 in Quil-A (Mahony et al., 2014; Mahony et al., 2015).

1.3.11 Conclusion

Despite availability of nearly 130 formulations, the use of commercial MLV and KV vaccines has not been able to control global BVDV prevalence in herds for the past 60 years (VIOLIN). Studies have consistently highlighted inadequate protective efficacy and safety concerns as major limitations of these vaccines. As a result, BVDV eradication programs do not depend solely on these vaccines. However, data from experimental BVDV vaccine studies have been instrumental in guiding the development of more effective contemporary vaccines. Consequently, the development of a novel broadly protective BVDV vaccine will greatly improve the management of BVDV and enhance productivity in cattle.

Vaccines that can confer broad protection against diverse BPI3V and BVDV strains ideally contain protective epitopes from all diverse circulating BPI3V and BVDV variants. Unlike currently approved or experimental vaccine candidates, including MLVs, which are based on

representative single-strain antigens, mosaic antigens incorporate epitopes from multiple strains and are thus suitable for developing broadly protective subunit vaccines. We hypothesize that live vectored vaccines expressing mosaic BPI3V and BVDV antigens, designed from all available sequenced BPI3V and BVDV genomes, would confer broad protection. This Thesis focuses on developing a live-attenuated vector for intranasal delivery of novel mosaic BPI3V and BVDV antigens and evaluating the safety, immunogenicity, and protective efficacy of recombinant prototype vaccines.

1.4 References

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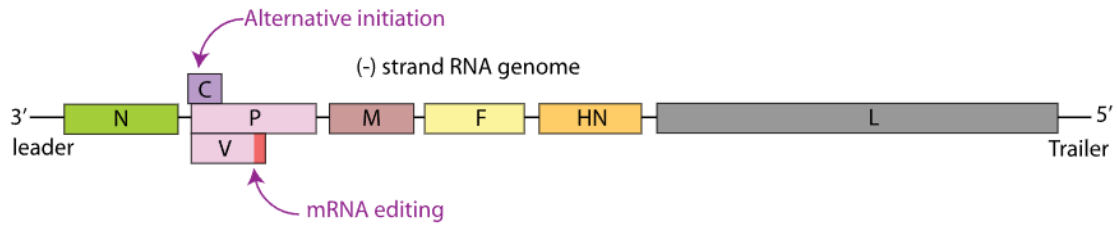


Figure 1.1. BPI3V genome organization.

Single-stranded, non-segmented, negative-sense RNA genome encoding six structural proteins: Nucleoprotein (N), Phosphoprotein (P), Matrix (M), Fusion (F), Hemagglutinin-Neuraminidase (HN), and Large Polymerase (L) and non-structural proteins (V, C and D). (Adapted from Viral Zone 2023 Swiss Institute of Bioinformatics).

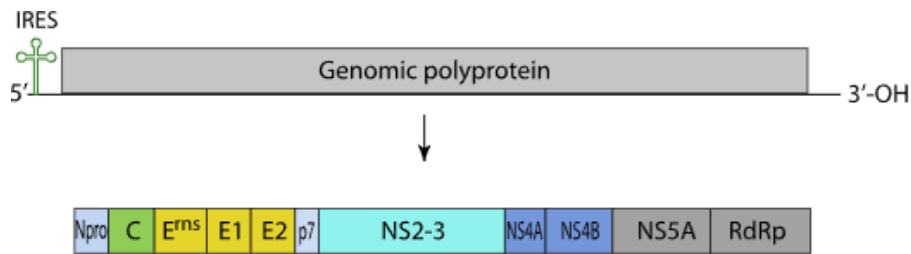


Figure 1.2. BVDV genome organization

BVDV single-stranded RNA genome. Precursor polyprotein undergoes proteolytic processing resulting in structural proteins (N^{pro}, capsid protein, E^{ns}, E1, and E2, glycoproteins, and non-structural proteins: N^{pro}, p7, NS2-3, NS4A, NS5A and NS5B (RdRp). (Adapted from Viral Zone 2023 Swiss Institute of Bioinformatics).

Chapter 2- BPI3Vc-vectored Surface Displayed Fusion and Hemagglutinin-Neuraminidase mosaic Antigens Elicit Broadly Neutralizing Antibodies in Cattle

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Abstract

Bovine Parainfluenza-3 virus contributes to Bovine Respiratory Disease Complex, causing severe pneumonia and death in cattle, leading to economic losses. Existing BPI3V commercial vaccines, based on genotype A strains, confer protection against some, but not all, genotype A strains and low neutralizing titers against genotypes B and C. This study aimed to develop a live vaccine capable of inducing broad protection against diverse BPI3V strains. Here, a BPI3V vaccine vector based on a genotype C strain was developed. A rescued recombinant BPI3V_{cmut}GFP virus exhibited a temperature-sensitive attenuated phenotype *in vitro*. Novel mosaic Fusion (F2) and Hemagglutinin-Neuraminidase (HN2) antigens, derived from consensus protein sequences of BPI3V genotypes A, B, and C, was used to develop a recombinant prototype vaccine, designated rBPI3V_{cmut}F2HN2. The recombinant virus replicated efficiently, displayed the mosaic antigen on the surface of infected cells, and remained stable over nine *in vitro* passages. Intranasal vaccination of calves with the rBPI3V_{cmut}F2HN2 virus induced strong systemic and mucosal IgG responses against BPI3V genotypes A, B, and C, which were significantly amplified upon boost, unlike the responses elicited by a commercial vaccine. Notably, sera from calves vaccinated with the rBPI3V_{cmut}F2HN2 virus produced significantly higher ($p < 0.0001$) neutralizing antibodies against BPI3V genotypes A-C compared to the commercial vaccine. The neutralizing antibodies were primarily generated by the mosaic F2HN2 antigens. Upon challenge with wild-type BPI3V genotype C virus, the rBPI3V_{cmut}F2HN2-vaccinated calves shed the least amount of virus in nasal fluids, had lower viral loads in their blood, and exhibited minimal pulmonary lesions. Therefore, rBPI3V_{cmut}F2HN2 virus is a promising vaccine candidate that has potential to confer broad protection against multiple BPI3V strains.

2.1 Introduction

Bovine Parainfluenza 3 Virus (BPI3V) is a respiratory pathogen that causes disease in cattle and is recognized as one of the etiological agents of Bovine Respiratory Disease Complex (BRDC), a leading cause of morbidity and mortality in feedlot cattle that results in economic losses (J. A. Ellis, 2010; Fulton et al., 2016). The virus was first isolated in the United States from nasal discharges of cows exhibiting respiratory disease symptoms (Reisinger RC, 1959). The BPI3V is classified within the family *Paramyxoviridae* and genus *Respirovirus*, with close antigenic and genetic relatedness to human parainfluenza 3 virus and Sendai virus (Abinanti & Huebner, 1959). The BPI3V has a single-stranded negative-sense RNA genome, approximately 16 kb in length, comprising six genes that encode for six structural proteins (Nucleoprotein N, Phosphoprotein P, Matrix M, Fusion F, Hemagglutinin-neuraminidase HN, and Large/polymerase L) and three non-structural proteins (V, C, and D) (J. A. Ellis, 2010).

The BPI3V is classified into three genotypes: A, B, and C. While genotypes B and C were previously reported in other countries, they are now circulating in U.S. cattle herds (Fulton et al., 2017; Neill, Ridpath, & Valayudhan, 2015; Newcomer et al., 2017). Current commercial BPI3V vaccines in the U.S. are mainly based on live attenuated genotype A strains, including SF-4 and Kansas/15626/84, which were isolated more than twenty years ago (Bailly, Mcauliffe, Skiadopoulos, Collins, & Murphy, 2000; Bryson et al., 1999; Fulton et al., 2016; Neill et al., 2015; Thorsen, Sanderson, & Bittle, 1969). These vaccines are effective against some homologous, but not heterologous strains, and their efficacy declines with antigenic drift. Notably, the number of emerging genomic and antigenic variant field strains, genotypes B and C, was documented to be quite significant in U.S. cattle herds (Fulton et al., 2016; Neill et al., 2015; Newcomer et al., 2017). Importantly, reference sera raised against genotype A virus elicits neutralizing antibody titers

against some, but not all, genotype A field strains and very low titers against genotypes B and C (Neill et al., 2015). This suggests that there is a widening gap in genetic and antigenic variability, which is further exacerbated by the insufficient evidence supporting heterologous protection from recent vaccines (Masic et al., 2020), underscoring the critical need for development of contemporary vaccines capable of conferring broad protection (Fulton et al., 2017).

Vaccines that induce virus-neutralizing antibodies are crucial for protection (J. A. Ellis, 2010; Frank & Marshall, 1971; R. Marshall & Frank, 1971). Although calves receive initial protection through maternal antibodies in colostrum, these antibodies wane by approximately ten weeks of age, leaving calves vulnerable to infections. Therefore, vaccination is essential for long-term immunity (Dawson, 1966; R. G. Marshall & Frank, 1975). Natural BPI3V infections or vaccination can induce neutralizing antibodies (R. Marshall & Frank, 1971; Morein, 1972; Thorsen et al., 1969). Moreover, natural killer cells and cytotoxic T lymphocytes (CTLs) play a role in eliminating BPI3V-infected cells (Campos, Rossi, & Lawman, 1982). Notably, CTL activity is detected as early as 6-9 days post-infection, coinciding with virus clearance observed in the closely related Sendai virus. This suggests that CTLs play a role in reducing viremia (Bamford, Adair, & Foster, 1995; Hou, Doherty, Zijlstra, Jaenisch, & Katz, 1992). However, it is important to note that, CD8⁺ CTL responses are typically short-lived and less robust compared to virus-neutralizing antibody responses (Tao et al., 2000).

Intranasal delivery of live BPI3V vaccines is more effective than parenteral methods in eliciting neutralizing antibodies in respiratory secretions, thereby reducing viral shedding (Todd, 1975). While some recent BPI3V vaccines are administered subcutaneously (Masic et al., 2020) or intramuscularly (Jiaqi Zhang et al., 2024), these routes do not mimic natural BPI3V exposure and are, therefore, limited in eliciting mucosal-neutralizing antibodies (Fulton et al., 2017).

Furthermore, using colostrum-deprived calves to conduct efficacy studies (Masic et al., 2020) may sometimes obscure maternally derived antibodies' inhibitory influence on immune response development, especially in calves under six months of age. High concentrations of mucosal, but not serum, antibody levels is associated with protection from clinical disease following challenge and thus are essential in mitigating initial infection (Gates et al., 1970; R. Marshall & Frank, 1971). Notably, certain commercially licensed BPI3Va based modified live virus (MLV) vaccines are administered intranasally to circumvent interference from BPI3V-specific maternal antibodies (Flynn et al., 2024; Metcalfe et al., 2020; P Nuijten, Cleton, Loop, Makoschey, & Vertenten, 2019; van Rooij et al., 2023).

Rationally designed live vectored subunit vaccines present an attractive platform for developing broadly protective BPI3V vaccines that can be updated to cover emerging strains of interest. These vaccines are characterized by their high immunogenicity, ability to stimulate both cellular and humoral immune responses, suitability for intranasal administration, and are not vulnerable to maternal antibodies. The F and HN surface glycoproteins are the main BPI3V immunodominant antigens and contain protective neutralizing epitopes, making them suitable targets for vaccine development (Caswell & Williams, 2016; Crowe JE, 1997; Haanes, Guimond, & Wardley, 1997). These targets have been utilized to develop subunit vaccines (Braun, Karvonen, King, Yoo, & Babiuk, 1999; Haanes et al., 1997; Jiaqi Zhang et al., 2024). It has also been reported that calves develop antibody responses against F and HN antigens one week after experimental aerosol infection, and a strong recall response occurs upon re-exposure. Serum antibody titers against these antigens correlate with protection (Toth & Frank, 1988). In humans, only the Parainfluenza 3 virus F and HN, but not other antigens, elicit antibodies capable of *in vitro* and *in vivo* neutralization (Kasel, Frank, Keitel, Taber, & Glezen, 1984). Importantly, F and HN antigen-

based subunit vaccines induce protective neutralizing antibodies that confer protection upon challenge (Durbin et al., 1999; Haanes et al., 1997; Jiaqi Zhang et al., 2024; Zhang et al., 2023). We hypothesized that a prototype live-vectored vaccine expressing mosaic F2 and HN2 antigens, designed using data from all sequenced BPI3V genotypes A-C and delivered intranasally, would confer broad protection.

In this study, a contemporary live attenuated BPI3Vc virus vector was developed for intranasal delivery of the novel mosaic F2 and HN2 antigens. Safety, tolerability, immunogenicity, and protective efficacy of the recombinant prototype vaccine, designated rBPI3Vc_{mut}F2HN2, was evaluated in Holstein calves challenged with wild-type BPI3Vc virus. Broad neutralization of representative wild-type BPI3V genotypes A, B, and C viruses was evaluated *in vitro*. BPI3V genotype A has previously been used as a vaccine vector, and it retains its infectivity and immunogenicity. It can, therefore, elicit immune responses against self as well as transgene-encoded antigens (Aurelia A Haller, Mitiku, & MacPhail, 2003; Karron et al., 1995; Schmidt, McAuliffe, Murphy, & Collins, 2001). The choice of BPI3V genotype C as a vector was informed by: i) the emergence of BPI3Vc strains in the U.S cattle herds; ii) absence of a protective BPI3Vc vaccine given that the currently available BPI3Va vaccine is poor at conferring cross-protection; and iii) availability of the BPI3Vc genome sequence (Fulton et al., 2017; Neill et al., 2015). Novel mosaic antigens, designated F2 and HN2, derived from the consensus of all F and HN protein sequences from the sequenced BPI3Va-c genomes in the U.S. as of 2019, were rationally designed for multicistronic expression and surface display on infected cells for optimal B-cell recognition. The rBPI3Vc_{mut}F2HN2 virus was evaluated for attenuation, virus stability, multicistronic antigen expression, surface display of the mosaic antigens, immunogenicity, and protective efficacy in calves following challenge.

2.2 Materials and Methods

2.2.1 Cells

Baby Hamster Kidney cells constitutively expressing T7 RNA polymerase (BSR T7/5 cells) (Buchholz, Finke, & Conzelmann, 1999), (kindly provided by Dr. Ursula J Buchholz, NIH), were maintained in Glasgow Minimum Essential Medium (Thermo Fisher Scientific) supplemented with 10 % Fetal bovine serum (FBS), 2 % MEM Amino Acid Solution (Thermo Fisher Scientific) and 1% GlutaMax (Thermo Fisher Scientific). Expression of T7 RNA polymerase was maintained by 1 mg/ml Geneticin (Thermo Fisher Scientific) selection on every second passage. Madin–Darby bovine kidney (MDBK) cells (Millipore Sigma, MDBK NBL-1) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% FBS, 1% GlutaMAX and 1% Non-essential amino acid (Thermo Fisher Scientific).

2.2.2 Wild-type BPI3V viruses

The BPI3Va strain Kansas/15626/84 GenBank: AF178654.1 was obtained from the National Institute of Health (kindly provided by Dr. Munir). The wild-type BPI3Vb strain TVMDL15 GenBank: KJ647284.1 and BPI3Vc strain TVMDL16 GenBank: KJ647285.1 were obtained from Texas A&M Veterinary Medical Diagnostic Laboratory. Viruses were propagated in MDBK cells at 37°C.

2.2.3 Monoclonal and polyclonal antibodies

Mouse anti-Flag tag monoclonal antibody (mAb) specific to the Flag epitope (DYKDDDDK) was commercially sourced (Millipore Sigma, F1804) and used to detect Flag-tagged F2 protein. Mouse anti-His tag mAb targeting the hexa-Histidine epitope (HHHHHH) was also commercially sourced

(Invitrogen, MA1-21315) and used to detect His-tagged HN2 protein. The Bovine Parainfluenza-3 virus antiserum was sourced from National Veterinary Services Laboratories (reagent code 470-BDV), and polyclonal IgGs from the sera were purified in-house by protein-G affinity chromatography (Invitrogen, 10-1242). Rabbit anti-Bovine Parainfluenza-3 virus genotype-C-specific antiserum was generated by rabbit hyperimmunization (out-sourced services from Robert Sargeant, Infinitex, CA) with wild-type BPI3Vc TVMDL 16 virus heat-inactivated at 56°C for 30 minutes. Polyclonal IgGs from the rabbit sera were purified by protein-G affinity chromatography and validated for binding to BPI3Vc-infected MDBK cells as judged by Immunocytometric analysis. For Immunofluorescence and Immunocytometric analyses, 4 µg/ml of monoclonal antibodies and 6 µg/ml of purified polyclonal IgG antibodies (pAbs) were used. For Western blots, 1.5 µg/ml of monoclonal antibodies were used.

2.2.4 Design and generation of BPI3Vc plasmid constructs

To design an attenuated BPI3Vc virus backbone, we selected the BPIV3c strain TVMDL16 GenBank: KJ647285.1 reference strain in the U.S. (Fulton et al., 2017), then compared its genome, *in silico*, to the genome of the current attenuated BPI3Va vaccine virus strain (Kansas/15626/84) and the defined mutations responsible for attenuation were introduced into the BPI3Vc backbone (Bailly et al., 2000; Aurelia A. Haller, MacPhail, Mitiku, & Tang, 2001; Skiadopoulos et al., 1999). The modified antigenomic sequence was used to generate a synthetic gene that was cloned into the pFLC plasmid vector to generate a construct, designated pFLC-BPI3Vc_{mut} (GenScript), that was used as the backbone for rescue of attenuated virus. This backbone was further modified to create a cloning site for transgenes in the non-coding region downstream of the N gene by using primers which introduced an AscI restriction site (ACCAGGCGCGCGCCAGC) (Fig. 2.1). Novel

mosaic BPI3V Fusion and Hemagglutinin-Neuraminidase genes, designated F2 and HN2, respectively, were designed and validated for protein expression. Briefly, sixty-two BPI3V genotypes A-C sequences encoding the F protein and thirty-five BPI3V genotypes A-C sequences encoding the HN protein were retrieved from NCBI, representing all the BPI3V genotypes A-C F and HN protein sequences that were available. Multiple sequence alignment was conducted using MEGA 7, which generated consensus sequences for both polypeptides based on a >50% identity threshold. In cases where identity was less than 50%, the most dominant amino acid was selected. If there was neither a consensus nor a dominant amino acid, residues from the BPI3Vc were selected. The final consensus sequences for the mosaic F2 and HN2 contained conserved polypeptide sequences from the annotated BPI3V genotypes A-C genomes and were included in generating maximum likelihood phylogenetic trees. A multicistronic expression cassette was designed to include the F2 and HN2 protein sequences separated by a 2A autocleavable motif (Ryan, King, & Thomas, 1991). A Flag tag was added, in-frame, at the N' end of the F2 polypeptide and a His tag was added, in-frame, at the C' end of the HN2 polypeptide (Fig. 2.3A). The resulting polypeptide was used to generate a synthetic multicistronic expression gene cassette that was codon-optimized for expression in *Bos taurus* and cloned into the pCDNA 3.1 (+) mammalian expression vector to generate a construct designated pCDNA-F2HN2 (GenScript). Expression of the mosaic F2 and HN2 antigens was validated by immunocytometric analysis using Alkaline phosphatase (AP)-conjugated anti-Flag mAb (Millipore Sigma, F3165), or mouse anti-His mAb (Invitrogen, MA1-21315) followed by an AP-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch, 115-055-003). Authenticity of the expressed antigens was similarly confirmed, but by using BPI3V antiserum 470-BDV (National Veterinary Services Laboratories) to probe the

transfected cells followed by an AP-conjugated goat anti-bovine IgG (Jackson ImmunoResearch, 101-055-003).

The genes encoding GFP, F2HN2, and an irrelevant *Theileria parva* modified sporozoite protein (TMSP) antigen (Tebaldi et al., 2017) were subcloned into the pFLC-BPI3V_{cmut} backbone to generate recombinant plasmid constructs, designated pFLC-BPI3V_{cmut}GFP, pFLC-BPI3V_{cmut}F2HN2, pFLC-BPI3V_{cmut}TMSP, respectively, for virus assembly. Briefly, the following primers were used to PCR the gene encoding GFP; Forward-1: 5' ATGGGATCCACGCGTGCCGCCGCCGCGCCATGGCCATGGTGAGCAAGGGCGCCGAGCTG TTC 3'; Forward-2: 5' TAAGAAAACTTAGGATTAACGGAGCCGCCACCATGGGATC CACGCGTGCCGCCGCCGC 3'; Forward-3: 5'GATTCTCGTATCGTATCTGGCGCGCC AAGTAAGAAAACTTAGGATTAACGGAGCCGC 3'; and GFP Reverse: 5'CGTGATA GTGGCGCGCCGCTAGCTATAGCGGCCGCATTACTATCACTTGTACAGCTCATC 3'. The following primers were used to PCR the gene encoding the F2HN2 multicistronic expression cassette: Forward-1: 5' CGCCAAGTAAGAAAACTTAGGATTAAC GGAGCCGCCACCATGGGATCCGCCGCCGCGCCATGGCCATGGCCACCATGATCACCATAG TTG 3'; F2HN2 Forward-2: 5' GATTCTCGTATCGTATCTGGCGCGCCAAGTAAGAAA AACTTAGGATTAACGGAGCCGC 3'; and F2HN2 Reverse: 5' CGTGATAGTGGCGCGCCG CTAGCTATAGCGGCCGCATCATTACTAGTGGTGATGGTGATG 3'. The TMSP gene was amplified using the following primers; TMSP Forward-1: 5' ACTGTGATAATAGTACGCGTGC CGCCGCCGCCATGGCCATGGCCATGACCACCATAGTTG 3'; Forward-2: 5' TAAGAAAA ACTTAGGATTAACGGAGCCGCCACCATGGGATCCACGCGTGCCGCCGCCGC 3'; Forward-3: 5' GATTCTCGTATCGTATCTGGCGCGCCAAGTAAGAAAACTTAGGATTA ACGGAGCCGC 3'; TMSP Reverse-1: 5'AGCTGTAGTGATAATAGTGCGGCCGCATTATC

ATTACTAGTGGTGATGGTGATGATG 3', and Reverse-2 5' CGTACTGGCGCGCCGCTAG CTATAGCGGCCGCATTATCATTACTAGTGGTGATGGTGATG 3'. These primers introduced an AscI restriction site at 5' end, N gene-end, Intergenic region (CTT), P Gene-start, and Kozak sequences upstream of the transgene and an AscI restriction site at the 3' end of the transgene (Fig. 2.3A). The primer design factored in requirements to meet the 'rule of six' by the recombinant antigenome sequence, which is critical for successful BPI3V virus assembly and efficient viral replication (Kolakofsky et al., 1998). A previously synthesized pcci-Brick BPI3V_{cwt}GFP construct (GenScript), the best expressing pCDNA-F2HN2 subclone, and a previously validated pCDNA-TMSP67 construct (GenScript) served as PCR templates, respectively. The PCR products were digested with AscI restriction enzyme, gene-cleaned, and subcloned into the AscI restriction site of the pFLCBPI3V_{cmut} plasmid backbone (Kolakofsky et al., 1998). Following transformation of MAX Efficiency™ Stbl2™ Competent Cells (Invitrogen, 10268019), colonies were screened by PCR using vector-specific Forward primer (5' AGGGCAGCCTGAATCCAGAGGAGATCAGGATCA 3') and gene-specific reverse primer GFP Reverse primer: 5'CGTGATAGTGGCGCGCCGCTAGCTATAGCGGCCGCATTACTAT CACTTGTACAGCTCATC 3'; F2HN2 Reverse primer: 5' CGTGATAGTGGCGCGCCGCTA GCTATAGCGGCCGCATCATTACTAGTGGTGATGGTGATG 3'; or TMSP Reverse-1: 5'AGCTGTAGTGATAATAGTGCGGCCGCATTATCATTACTAGTGGTGATGGTGATGATG 3'; to identify positive constructs. The presence of transgenes in plasmid minipreps of selected positive constructs was confirmed by AscI digest followed by Sanger sequencing validation.

2.2.5 Generation and validation of viruses

To rescue viruses, helper plasmids were first generated and validated for protein expression *in vitro*. Briefly, genes encoding N, P, and L proteins were PCR amplified from the above mentioned pcci-Brick BPI3V_{Cwt}GFP (GenScript) construct encoding the wild-type BPI3Vc virus backbone, using primers that introduced a Flag tag at the 3' end, i.e. (N Forward: 5'ATATACCGCGACA GAATCAAGCTTGCCGCCACCATGTTGAGTCTGTTTGATACATTC 3' and N Reverse: 5' ATGACGATAAGTCCTCGAGCTATTACTTATCGTCATCGTCCTTGTAGTCGCTACTTCCA 3'; P Forward: 5'ATATACCGCGACAGAATCAAGCTTGCCGCCACCATGGAAGACAA TGTTCAAAACAATC 3' and P Reverse: 5' ATGACGATAAGTCCTCGAGTTACTACTTATC GTCATCGTCCTTGTAGTCTTGGGAGCTAA 3'; L Forward: 5'ATATACCGCGACAGAAT CGCGGCCGCGCCGCCACCATGGACACCGAATTCAGCGGTGG 3' and L Reverse: 5' TGA CGATAAGTCCTCGAGCTATCATTACTTATCGTCATCGTCCTTGTAGTCATCAATATC 3'). The amplified PCR products were subcloned into pCDNA 3.4 TOPO (Invitrogen) to generate pCDNA-N, -P and -L constructs, respectively. Protein expression was evaluated by immunocytometric analysis using an anti-Flag mAb conjugated to Alkaline Phosphatase (Millipore Sigma) or the above-mentioned rabbit anti-BPI3Vc pAb. To assemble virus, 3µg of the antigenome plasmids pFLC-BPI3V_{Cmut}, pFLC-BPI3V_{Cmut}GFP, pFLC-BPI3V_{Cmut}F2HN2 or pFLC-BPI3V_{Cmut}TMSP, along with 1.6µg of pCDNA-N, 1.6 µg of pCDNA-P and 0.8 µg of pCDNA-L helper plasmids were co-transfected into the BSR T7/5 cells in 6-well plates using Lipofectamine 3000 reagent (3.75 µl of the Lipofectamine 3000 reagent and 5 µl of P3000 reagent, Invitrogen, L3000015). Cultures were incubated at 37°C for three days, then co-cultured with MDBK cells at a ratio of 1:4 and monitored daily for virus assembly. Rescued viruses were identified by observing cytopathic effects (CPE) 5-10 days later, and then virus stocks were processed from cell pellets

and supernatants. Protein expression by the rescued viruses was validated by immunocytometric assay as above or by immunofluorescence assay (IFA). Briefly, MDBK cells were grown in 12-well plates and infected in triplicates with 10-fold diluted stock rescued BP3V_{cmut} or the recombinant BPI3V_{cmut}F2HN2 virus (rBPI3V_{cmut}F2HN2) for 48-72 hrs. at 37°C. The infected cells were then fixed and probed with the mouse anti-Flag mAb (Millipore Sigma), the mouse anti-His mAb (Invitrogen), or the rabbit anti-BPI3Vc pAb. For IFA, FITC-conjugated secondary antibodies were used at 1:1000 dilution, including goat anti-mouse IgG (Jackson ImmunoResearch) or goat anti-rabbit IgG (Jackson ImmunoResearch). Non-infected cells were used as negative controls. Expression of GFP by the recombinant BPI3V_{cmut}GFP (rBPI3V_{cmut}GFP) virus was evaluated using an Olympus CKX53 fluorescent microscope, and images were captured using an AmScope MU1803-HS digital camera. Protein expression was used to select one best rBPI3V_{cmut}F2HN2 clone, as judged by IFA, which was expanded in MDBK cells and the virus was purified by sucrose gradient ultra-centrifugation at 20,000 rpm for 2 hrs. over a 30%-70% sucrose gradient. The recovered virus was dialyzed twice in 1x PBS, aliquoted, snap-frozen, and then stored at -80°C.

Expression of the individual Flag-tagged F2 (~60 kD) and the His-tagged HN2 (~64.5 kD) antigens by the purified rBPI3V_{cmut}F2HN2 virus encoding the multicistronic expression cassette was confirmed by Western blot analysis. The wild-type virus BPI3Vc TVMDL16 was used as a negative control. Briefly, approximately 16 µg of the purified rBPI3V_{cmut}F2HN2 virus was prepared under reducing conditions using NuPage LDS sample buffer (Invitrogen) and NuPage sample reducing buffer (Invitrogen), following the manufacturer's instructions. A 30 µl aliquot of the reduced and denatured sample was resolved on a 4-12% NuPAGE® Bis-Tris gel (Invitrogen) and then transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked with Starting

Block™ Blocking Buffer (Invitrogen) and probed with 1.5 µg/ml HRP-conjugated anti-Flag mAb (Sigma-Aldrich), or 1.5 µg/ml mouse anti-His mAb (Invitrogen), followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) diluted at 1:1000 in blocking buffer. Protein bands were visualized by Chemiluminescence using the Clarity Western ECL substrate (Bio-Rad).

2.2.6 Virus Growth curves

MDBK cells were grown in six-well plates to 80% confluency and then infected in duplicates at a Multiplicity of infection (MOI) of 1 with the following viruses: i) BPI3V_{cwt}virus (TVMDL16); ii) rBPI3V_{cmut}GFP virus; or iii) rBPI3V_{cmut}F2HN2 virus. The infected cells were incubated at 37°C, 39°C, or 41°C. Every 24 hrs., cultures were gently mixed, and a 0.5 ml sample was taken and replaced with an equal volume of fresh media. For the temperature-sensitive assay, sampling was done on days 1, 2, and 3. Virus aliquots were snap-frozen and stored at -80°C. Virus titers were determined for each of the time points by TCID₅₀ calculation using the Reed–Muench Method (Lei, Yang, Hu, & Sun, 2021). For the multicycle replication growth assay, a similar setup was carried out and sampling was done on days 1-4.

2.2.7 Flow cytometric analysis

The F2 and HN2 polypeptide sequences were designed to retain their native transmembrane domains, enabling anchoring of the extracellular F2 and HN2 domains on the viral surface, thus mimicking the native presentation of F and HN glycoproteins on the virus, allowing their surface display during replication and packaging of viral progeny in permissive cells. The Flag and His tags that were added, in-frame, to F2 and HN2, respectively, allowed differentiation of the mosaic transgene products from their corresponding wild-type F and HN proteins in the BPI3Vc

backbone. Surface expression of the F2 ectodomain by rBPI3V_{cmut}F2HN2-infected MDBK cells was analyzed by Flow cytometry. Briefly, MDBK cells were grown to 80% confluency, then infected with either the rBPI3V_{cmut}F2HN2 virus or an irrelevant recombinant BPI3V_{cmut}SARS-CoV-2-Spike (rBPI3V_{cmut}Spike) virus (generated in-house as a COVID-19 prototype subunit vaccine), at an MOI of 1. After incubation at 37°C for 48 hrs., the infected cell monolayer was washed in 1x PBS, trypsinized using 1x TrypLE select enzyme (Invitrogen), and prepared for surface staining. The cells were aliquoted into 1.0 x10⁶ cells per tube and washed twice with 2% FBS in PBS (blocking buffer). The cells were blocked for 30 minutes at 4°C and then incubated with mouse anti-Flag DyLight 488 mAb (1:250, spectrally similar to AF 488) (Invitrogen, MAI-91878-D488) for 30 minutes at 4°C in the dark. Afterward, the cells were washed twice and resuspended in the blocking buffer. Data was acquired using Attune™ NxT Acoustic Focusing Flow Cytometer (Invitrogen, A24863). The data was analyzed using FlowJo v10. Analysis and gating were guided by data from the negative control irrelevant rBPI3V_{cmut}Spike sample, which did not contain any tag.

2.2.8 Viral RNA extraction and sequencing

MDBK cells were grown in six-well plates to 80% confluency and then infected with the rescued rBPI3V_{cmut}F2HN2 Passage 1 (P1) virus at an MOI of 1. Infected cells were incubated at 37°C for four days, and the resulting P2 virus was used to infect fresh MDBK cells at a ten-fold dilution. After nine passages, the cell pellets of P2 virus and P9 virus, were collected in 1 ml of Trizol (Thermo Fisher Scientific, 15596026) and processed for total RNA isolation following the manufacturer's instruction. Briefly, phase separation was achieved using 0.2 ml Chloroform (Ambion, AM9730), followed by isopropanol precipitation and ethanol washes. Purified RNA was

resuspended in RNase-free water, reverse transcribed to cDNA using Sequenase 2.0 DNA Polymerase (Life Technologies, 70775Z), Superscript III First Strand Synthesis System (Invitrogen, 18080051) and TaKaRa Taq DNA Polymerase (Clontech, R001a). Sequencing libraries were prepared using the NexteraXT DNA Library Preparation Kit (Illumina, FC-131-1096) and Nextera XT DNA Library Preparation Index Kit (96 Indexes, 384 Samples) (Illumina, FC-131-1002), following the manufacturer's instructions. Libraries were quantified using a Qubit Flex (ThermoFisher, Q33327). Sequencing was performed on the Illumina MiSeq platform, (Illumina, SY-410-1003), generating paired end reads. Raw sequencing data were processed for quality control and trimming using the Trim Reads function in CLC Genomics Workbench Premium Desktop (Qiagen, 832023), followed by alignment to the reference genome using CLC Genomics Workbench Premium Desktop. The identity percentage of each gene was obtained by alignment to reference gene and calculation of pairwise comparison. Genomic and Read coverage data was obtained from the Quality control output of the mapped reads.

2.2.9 Calf immunization

Fifteen male Holstein calves (five months old, sourced from J.R. Livestock Inc, Iowa) were confirmed to be seronegative for BPI3V as determined by sera neutralization tests conducted by Iowa State University Veterinary Diagnostic Laboratory. The calves were then randomly allocated to three groups (n=5) (Table 2.2). Seven days after acclimatization, each calf in group 1 received a priming dose of the rBPI3V_{cmut}F2HN2 prototype vaccine (5.4×10^8 TCID₅₀) followed by a 1st booster dose of 2.5×10^{10} TCID₅₀, and a second booster 2 dose of 4.4×10^{10} TCID₅₀ at weeks 3 and 6 post-priming, respectively (Fig. 2.6A). The calves were immunized via intranasal administration of 2 ml of the virus (diluted in Dulbecco's Modified Eagle Medium, DMEM) using

an Intranasal mucosal atomization device (Teleflex MAD 300). Calves in group 2 were immunized with a commercial bivalent IBR/PI3 modified live vaccine Bovilis® Nasalgen® IP (Merck Animal Health, Intervet Inc.), administered at the recommended 2 ml dose. Calves in group 3 served as negative controls and they received 2 ml of DMEM for both the prime and booster doses. The rBPI3V_{cmut}F2HN2 virus immunogen was back-titrated after administering the priming and booster doses. During the immunization phase, the three groups of calves were housed separately. Their body weights and rectal temperatures were recorded weekly.

2.2.10 Evaluation of antibody responses

Coagulated blood and nasal fluids were collected at various time points (Fig. 2.6A) and Indirect ELISA was used to evaluate antibody responses. Briefly, sera were obtained from the coagulated blood by centrifugation, while nasal fluids were collected in 500 µl of 1x PBS resuspension. 96 well polystyrene ELISA microplates (Nunc, Thermo Fisher Scientific, 80040LE 0910) were coated in triplicate with 100 µl of heat-inactivated BPI3Va (Kansas strain), BPI3Vb (TVMDL 15 strain), or BPI3Vc (TVMDL16 strain) virus diluted in sodium bicarbonate buffer at final concentration of 1 µg/ml, and the plates were incubated at 4°C overnight. The plates were washed with PBS-Tween 20 (PBST) and then blocked with 300 µl of 1% BSA/PBST for one hr. at 37°C. Following washes, 100 µl of sera diluted 1:100 in the blocking buffer was added in triplicates and incubated for one hr. at 37°C. After washing unbound primary antibodies, 100 µl of HRP-conjugated goat anti-bovine IgG (Jackson ImmunoResearch, 101-035-003) diluted at 1:5000 in blocking buffer was added and the plates were incubated for one hr. at 37°C, washed and developed using 100 µl One Step Ultra TMB (Thermo Fisher Scientific, 34029). The reaction was stopped after ten minutes using 100 µl of 1N Hydrochloric acid and read at 450 nm using a BioTek microplate reader

(Synergy H1 Multi-mode reader). BPI3V-specific IgG responses in each group were presented as mean Optical density values. BPI3V-specific IgG responses in nasal fluids were similarly determined, but at 1:4 sample dilution.

2.2.11 Virus Neutralization

Neutralization of wild-type BPI3Va (Kansas strain), BPI3Vb (TVMDL 15 strain), and BPI3Vc (TVMDL16 strain) viruses was evaluated using sera from blood collected from the study animals one week after the last boost. Briefly, sera samples were heat-inactivated at 56°C for 30 minutes and 50 µl of each sera sample was serially diluted two-fold in 96-well microtiter plates using DMEM for triplicate test wells. The BPI3Va-c virus stocks were diluted to 300 TCID₅₀/ml, and 50 µl of this diluted virus was added to each test well. The serially diluted sera were then incubated with the virus for two hrs. at 37°C, and then 10,000 MDBK cells/well were added in a volume of 100 µl. The plates were incubated at 37°C for 72 hrs. and monitored daily for signs of CPE. The presence or absence of virus was confirmed by observing CPE and by IFA using the rabbit anti-BPI3Vc pAb. Virus neutralization titers were expressed as the reciprocal of the highest dilution in which more than 50% CPE or fluorescent staining for the virus was detected (Newcomer et al., 2017; Zhang et al., 2023). Sera obtained from age-matched Holstein calves, undergoing a similar immunization regimen, but with a cognate irrelevant virus construct, rBPI3Vc_{mut}TMSP, was used as the control for neutralization assays.

2.2.12 Animal Challenge

At sixty days post-priming, the calves were challenged by intranasal instillation of 2.9×10^7 TCID₅₀ dose of the wild-type BPI3Vc TVMDL16 virus (challenge virus was back-titrated after

administration) (Thorsen et al., 1969). The calves were monitored daily for 21 days for clinical signs of respiratory disease, including pyrexia ($\geq 103^{\circ}\text{C}$), coughing, mucopurulent nasal discharge, ocular discharge, loss of appetite, and depression as previously described (J. A. Ellis, 2010). Body weights were recorded on days six and twelve post-challenge, while nasal swabs and uncoagulated blood samples were collected every three days for 18 days. Eight days post-challenge, one calf from the rBPI3Vc_{mut}F2HN2 group, 5661, died due to conditions unrelated to the study (bowel perforation). Three weeks later, the calves were humanely euthanized and transported for necropsy following approved protocols to the Kansas State Veterinary Diagnostic Laboratory.

2.2.13 Hematology, Nasal and blood viremia

Uncoagulated blood was collected in K2 EDTA-coated tubes on days 0, 3, 6, 9, 12, 15, and 18 post-challenge to evaluate white blood cell counts, platelet counts, and viremia in blood. Blood samples were gently inverted several times after collection, and a Complete Blood Count analysis was performed using an automated hematology analyzer Siemens Advia 2120 at Kansas State University Veterinary Diagnostic Laboratory. The White Blood Cell (WBC) and Platelet (PLT) counts were recorded and verified by two staff personnel. To establish a common baseline, the mean WBC or Platelet count for each group on DPC 3, 6, 9, 12, 15, and 18 were subtracted from their respective initial values on DPC 0, and the resulting differences were plotted as mean count ratios relative to baseline.

Post-challenge wild-type BPI3Vc virus titers in nasal fluids and blood were determined by sample titration in MDBK cells followed by IFA as previously described (Piet Nuijten et al., 2022; Vangeel, Ioannou, Riegler, Salt, & Harmeyer, 2009; Xue et al., 2010). Briefly, nasal swabs were collected in 500 μl 1x PBS supplemented with 1% Penicillin-Streptomycin antibiotic, vortexed,

centrifuged, filter sterilized and diluted five-fold in DMEM. Blood samples were subjected to three freeze-thaw cycles, centrifuged, and the lysate diluted ten-fold in DMEM. 50 µl of serially diluted nasal fluid samples or cell lysate samples were used to infect 96-well MDBK cell monolayers in quadruplicates (for swabs) or nine wells (for lysates). The plates were incubated at 37°C for five days and then fixed with formalin for staining using the rabbit anti-BPI3Vc pAb mentioned above and a FITC-conjugated goat-anti Rabbit IgG (Jackson ImmunoResearch). The highest sample dilution where positive MDBK cell staining was observed was used to report the BPI3V titer, as previously reported (Brock, Grooms, Ridpath, & Bolin, 1998; Lokhandwala et al., 2017; Sangewar et al., 2020). Due to limited amount of nasal fluid sample, the endpoint viral dilution titer and not the standard TCID₅₀/ml titer determination was conducted.

2.2.14 Necropsy and pathology

To terminate the study, the animals were sedated with xylazine and euthanized by penetrative captive bolt at the study endpoint, and complete necropsy examinations were directed by board-certified pathologists who were blinded to the animal groupings. All tissues were examined for evidence of abnormalities, and all gross lesions were recorded, and digital photographs were taken. In addition, digital photographs were collected from all animals of thoracic cavity with thoracic organs *in situ*, and the bilateral lungs after removal from thoracic cavity. The lungs were sampled systematically, and sections of lung that included bronchi were collected and labelled prior to fixation in 10% neutral buffered formalin from the following sites: cranial, middle, and caudal lobes of the right lung and the cranial and caudal lobes of the left lung. The tracheobronchial lymph nodes were measured and recorded. The left cranial, left and right middle lung sections were routinely processed for histopathologic evaluation after 48 hrs. of formalin fixation. Hematoxylin

and eosin (H&E) staining of 4-micron sectioned paraffin-embedded tissues was performed by standard laboratory protocols in the histopathology laboratory of the Kansas State Veterinary Diagnostic Laboratory. All tissues were evaluated by two veterinary pathologists (KMB, BLP) and were scored using a modified lung scoring system adapted from previous studies (Chowdhury et al., 2021; J. Ellis et al., 2020). Briefly, all lung sections were given an appropriate morphologic diagnosis for each tissue section, which included the most significant observed histologic lesions: lymphoplasmacytic and histiocytic interstitial pneumonia; neutrophilic bronchitis and bronchiolitis; lymphoplasmacytic peribronchiolitis; bronchiolitis obliterans; and bronchial associated lymphoid hyperplasia. The severity from mild, moderate, to severe of each histologic lesion was noted. A summary morphologic diagnosis was then given for each calf based on individual lobe diagnoses, and numerical scores were assigned for each summary morphologic diagnosis by summing the individual lesion scores on a scale reflecting the presence and severity of each lesion within each calf summary morphologic diagnosis (0= normal; 1 = lesion is present; 2 = mild; 3 = moderate; 4 = severe).

2.2.15 Statistical analysis

Data was analyzed using GraphPad Prism 10. For the ELISA data analysis, the significance of differences in mean BPI3V-specific IgG responses between the treatment and control groups during the different sampling time points were analyzed by Two-way ANOVA, followed by Geisser-Greenhouse correction for sphericity and Tukey correction for multiple mean comparisons. For the virus neutralization, clinical outcomes, nasal viral shedding, and histopathology data analysis, the significance of differences between the treatment and control groups during the different time points were analyzed by Two-Way ANOVA followed by Tukey

correction for multiple mean comparisons. For the blood viremia analysis, the significance of differences between the treatment and control groups was analyzed by One-Way ANOVA followed by Dunnett's T3 correction for multiple comparisons. Statistical differences with significance levels of $p < 0.05$ was considered significant.

2.3 Results

2.3.1 Recombinant virus is temperature sensitive and express transgenes

A modified BPI3V genotype-C (BPI3Vc) virus backbone based on the TVMDL16 isolate that contain mutations responsible for attenuation of the current BPI3V genotype-A (BPI3Va) commercial vaccine was designed with an *AscI* restriction site in the non-coding region of the N gene for cloning of transgenes, and a synthetic gene was used to generate a plasmid construct designated pFLC-BPI3Vc_{mut} (Fig. 2.1A). A mutant virus, designated BPI3Vc_{mut}, was rescued and validated using convalescent polyclonal Ab (rabbit anti-BPI3Vc pAb) (Fig. 2.1B). The ~0.8 kb gene encoding GFP was subcloned into pFLC-BPI3Vc_{mut} plasmid (Fig. 2.1C) and a recombinant virus, rBPI3Vc_{mut}GFP, that was assembled using the resultant construct, pFLC-BPI3Vc_{mut}GFP, expressed the transgene (Fig. 2.1D). The virus displayed a temperature sensitive attenuated phenotype *in vitro* (Fig. 2.1E-G). Notably, replication of the rBPI3Vc_{mut}GFP virus was similar to the wild-type BPI3Vc TVMDL16 virus at 37°C, resulting in CPE with peak titers of 8.9 TCID₅₀/ml and 10.3 TCID₅₀/ml, respectively, (Fig. 2.1E-G). However, the replication efficiency of the rBPI3Vc_{mut}GFP virus declined at 39°C and reduced significantly at 41°C (Fig. 2.1E and G). In contrast, the onset of replication, CPE, and peak replication titers observed in the cells infected with the BPI3Vc wild-type virus remained relatively unchanged even at 41°C, with titers reaching 8.14 TCID₅₀/ml (Fig. 2.1E and F). Novel mosaic BPI3V Fusion and Hemagglutinin-

Neuraminidase polypeptides, designated F2 and HN2, respectively, based on consensus F protein sequence from sixty-two BPI3V genotypes A-C genomes and consensus HN protein sequence from thirty-five BPI3V genotypes A-C genomes, were generated (Fig. 2.2 A and D). The F2 mosaic polypeptide sequence displayed a 74.1% identity to all the 62 F protein sequences from the BPI3Va, b and c strains, while the HN2 mosaic polypeptide sequence was 77.5% identical to all the 35 HN protein sequences from the BPI3Va, b and c strains (Fig. 2.2 B and E). Importantly, the mosaic F2 sequence displayed 93.5% and 92.8% homology with the F sequence of BPI3Va SF-4 and Kansas/ 15626/84 vaccine strains, respectively (Fig. 2.2 C), whereas the mosaic HN2 sequence displayed 86.5% and 85.8% homology with the HN sequence of BPI3Va SF-4 and Kansas/ 15626/84 vaccine strains, respectively (Fig. 2.2 F). A codon-optimized synthetic gene encoding the F2 and the HN2 polypeptides, separated by the 2A auto-cleavable motif, was used to generate a plasmid construct, designated pCDNA-F2HN2. The construct expressed the encoded antigens that were recognized by anti-tag mAbs and more importantly, by BPI3V-specific serum (Fig. 2.3B). The ~3.6 kb gene encoding the F2HN2 multicistronic expression cassette was subcloned into pFLC-BPI3V_{cmut} plasmid (Fig. 2.3C) and used to generate a recombinant virus, rBPI3V_{cmut}F2HN2, which expressed the encoded proteins as judged by IFA and Western blot assay using Flag- and His-tag-specific mAbs and authenticity of the antigens was validated using rabbit anti-BPI3Vc pAb (Fig. 2.3D and E). Rescued rBPI3V_{cmut}F2HN2 virus, unlike an irrelevant negative control rBPI3V_{cmut}Spike, displayed the F2 antigen on the surface of MDBK infected cells (Fig. 2.4).

2.3.2 Recombinant virus is stable

Analysis of virus replication growth curves showed that the presence of transgenes did not inhibit virus replication (Fig. 2.5). Multicycle replication of the rBPI3Vc_{mut}F2HN2 virus in MDBK cells at 37°C was similar to that of the rBPI3Vc_{mut}GFP virus. By day four, the virus titer for the rBPI3Vc_{mut}F2HN2 was 8.27 log₁₀ TCID₅₀/ml, while rBPI3Vc_{mut}GFP had a 9.16 log₁₀ TCID₅₀/ml titer. Both titers were comparable to the 8.11 log₁₀ TCID₅₀/ml titer recorded for the wild-type BPI3Vc TVMDL16 virus (Fig. 2.5), suggesting that the presence of the ~0.8 kb GFP insert or the ~3.6 kb F2HN2 transgene did not affect overall virus replication.

To evaluate the stability of F2HN2 antigen expression in MDBK cells, the rBPI3Vc_{mut}-F2HN2 virus was serially passaged multiple times *in vitro* and the full-length viral antigenome of the progenies were sequenced. After nine passages, reads covering the N, P, M, F genes of the BPI3Vc backbone and the gene encoding the mosaic F2HN2 polypeptide remained identical to the original sequence and the data from P2 progenies. However, minor point mutations were identified in the HN gene (K254R) and L gene (S2220Y, S2221L) of passage nine virus (Table 2.1A-B). Therefore, passage of the rBPI3Vc_{mut}F2HN2 virus did not significantly alter the mosaic antigen sequence or the overall virus antigenome sequence, underscoring the stability of the vector *in vitro* and its potential suitability as a vaccine replicon.

2.3.3 rBPI3Vc_{mut}F2HN2 virus is safe and elicited systemic and mucosal antibody responses

The rBPI3Vc_{mut}F2HN2 virus was well tolerated by five-month-old Holstein calves following homologous prime-boost intranasal immunization, and no adverse clinical side effects were observed. The calves remained healthy, maintained relatively consistent body temperatures, and showed steady body weight gain during the immunization phase (Fig. 2.6A-C). Similar outcomes

were observed in the positive control calves which received the commercial Bovilis Nasalgen PI3 vaccine as well as the sham-treated negative controls (Fig. 2.6A-C). More importantly, the rBPI3V_{cmut}F2HN2 virus elicited strong BPI3V-specific systemic and mucosal IgG responses that were amplified after boosting (Fig. 2.7). Notably, two weeks post-priming, all the calves immunized with the rBPI3V_{cmut}F2HN2 virus or the Bovilis Nasalgen PI3 vaccine, but not the sham-treated calves, seroconverted (Fig. 2.7). The vaccinees had similar serum and mucosal IgG responses against heat-inactivated BPI3Va (Kansas strain), BPI3Vb (TVMDL 15 strain) and BPI3Vb (TVMDL16 strain) wild-type viruses (Fig. 2.7A-F). Compared to the sham controls, significantly higher ($p<0.0001$) BPI3Va-c-specific serum IgG responses were detected in the calves immunized with the rBPI3V_{cmut}F2HN2 virus two weeks after the first and the second boosts (Fig. 2.7A-C). Importantly, these IgG responses were significantly higher than the responses detected in the Bovilis Nasalgen PI3 vaccinees at the same sampling timepoints (Fig. 2.7A-C). Notably, following the first, but not the second boost, the BPI3Va-c-specific serum IgG responses primed by the rBPI3V_{cmut}F2HN2 virus were significantly amplified ($p<0.0001$, $p=0.0002$, and $p<0.001$, respectively) (Fig. 2.7A-C). Similar outcomes were observed in the Bovilis Nasalgen PI3 vaccinees, but boosting had a much lower effect (Fig. 2.7A-C).

Interestingly, intranasal immunization of calves with the rBPI3V_{cmut}F2HN2 virus primed BPI3Va-c-specific mucosal IgG responses that were significantly recalled after boosting (Fig. 2.7D-F). Specifically, compared to the sham treatment, the rBPI3V_{cmut}F2HN2 virus primed significant mucosal IgG responses against BPI3Va ($p=0.0043$), BPI3Vb ($p=0.0346$), and BPI3Vc ($p=0.0024$) (Fig. 2.7D-F). Similar to the outcome observed for serum IgG responses, the first, but not the second dose had significant booster effect on BPI3Va-c-specific mucosal IgG responses ($p=0.0829$, $p=0.0325$, and $p=0.0279$, respectively) (Fig. 2.7D-F). Even though the Bovilis

Nasalgen PI3 vaccine primed slightly higher BPI3Va-c-specific mucosal IgG responses compared to the responses primed by the rBPI3V_{Cmut}F2HN2 virus, boosting with the commercial vaccine did not have a booster effect (Fig. 2.7D-F). Overall, the rBPI3V_{Cmut}F2HN2 virus had significantly higher booster effect on both serum as well as mucosal IgG responses compared to the Bovilis Nasalgen PI3 vaccine (Fig. 2.7A-F). Surprisingly, the Bovilis Nasalgen PI3 vaccine had a subtle booster effect on serum, but not on mucosal IgG responses (Fig. 2.7A-F).

2.3.4 rBPI3V_{Cmut}F2HN2 prototype vaccine elicited broadly neutralizing antibodies

Immunization of calves with the rBPI3V_{Cmut}F2HN2 prototype vaccine induced antibody responses that neutralized wild-type BPI3Va (Kansas strain), BPI3Vb (TVMDL 15 strain), and BPI3Vc (TVMDL16 strain) viruses as judged by virus neutralizing assay (VNA) using sera from blood collected one week after the second boost (Fig. 2.8). Specifically, the rBPI3V_{Cmut}F2HN2 virus construct induced significantly higher ($p < 0.0001$) BPI3Va-c neutralizing antibody titers compared to the response detected in sera from the Bovilis Nasalgen PI3 vaccinees as well as the sham controls (Fig. 2.8A-C). Notably, the VN titers elicited against the wild-type BPI3Va-c viruses by the rBPI3V_{Cmut}F2HN2 prototype vaccine were 1:4096-1:8192; 1:2048-1:4096; and 1:4096-1:8192, respectively (Fig. 2.8A-C). In comparison, the VN titers elicited against the wild-type BPI3Va-c viruses by the Bovilis Nasalgen PI3 vaccine were 1:256-1:1024; 1:128-1:512; and 1:64-1:512, respectively (Fig. 2.8A-C).

2.3.5 Mosaic F2HN2 antigens were critical for eliciting neutralizing antibody

The BPI3Va-c neutralizing antibodies primed and expanded by the rBPI3V_{Cmut}F2HN2 prototype vaccine were mainly elicited by the mosaic F2HN2 antigens. Calves vaccinated with the

rBPI3Vc_{mut}F2HN2 vaccine elicited significantly higher virus neutralization titers against wild-type BPI3Va and c ($p < 0.0001$), as well as BPI3Vb ($p < 0.001$), compared to titers elicited by a cognate control virus construct, rBPI3Vc_{mut}TMSP, expressing an irrelevant *Theileria parva* sporozoite protein (TMSP) (Fig. 2.9). Notably, the neutralizing antibody titers observed in the sera from the rBPI3Vc_{mut}TMSP vaccinees against wild-type BPI3Va (1:64-1:512), BPI3Vb (1:128-1:1024), and BPI3Vc (1:512-1:2048), resembled the titers observed in the sera from the Bovilis Nasalgen PI3 vaccinees (Fig. 2.8).

2.3.6 Post-challenge clinical signs

Following intranasal challenge of all the calves with wild-type BPI3Vc TVMDL 16 virus, some calves developed one or more observable signs of clinical disease, including coughing, nasal discharge, ocular discharge, loss of appetite, depression, and pyrexia. However, no significant differences were observed between the treatment groups. Calves in all the treatment groups developed fever, with peak mean temperatures recorded on day 9 at 103°C, day 10 at 102.5°C, and day 11 at 102.8°C for the rBPI3Vc_{mut}F2HN2 vaccinees, the Bovilis Nasalgen PI3 vaccinees, and the sham treatment controls, respectively. There was no significant difference in temperature fluctuations between the vaccinated and the non-vaccinated negative controls (Fig. 2.10A). Post challenge, the mean weight of the rBPI3Vc_{mut}F2HN2 vaccine group increased from 456.2 lbs. to 504.5 lbs., a gain of 48.3 lbs.; while the mean weight of the Bovilis Nasalgen PI3 vaccinees increased from 467.2 lbs. to 521.5 lbs., a gain of 54.3 lbs. In contrast, the sham treatment controls had the lowest mean weight gain, from 430.8 lbs. to 474.4 lbs., a gain of 43.6 lbs. (Fig. 2.10B).

All treatment groups experienced a decrease in WBC counts, with onset detected on day 3 post-challenge in the rBPI3Vc_{mut}F2HN2 vaccinated calves and on day 6 post-challenge in the

Bovilis Nasalgen vaccinated calves and the sham-treated calves (Fig. 2.10C). The greatest drop in WBC counts happened on day 9 post-challenge, from 9,040/ μ l on DPC 0 to 6,700/ μ l in the rBPI3V_{cmut}F2HN2 vaccinees; 8,720/ μ l on DPC 3 to 8,380/ μ l in the Bovilis Nasalgen vaccinees; and 7,540/ μ l on DPC 3 to 6,860/ μ l in the sham-treated calves. Surprisingly, the rBPI3V_{cmut}F2HN2 vaccinees had the lowest mean WBC counts throughout the challenge phase compared to the Bovilis Nasalgen PI3 vaccinees and the negative controls (Fig. 2.10C). The Bovilis Nasalgen PI3 vaccinees had significantly higher mean WBC counts ($p=0.0371$) compared to the rBPI3V_{cmut}F2HN2 vaccinees on day 15 post-challenge (Fig. 2.10C). This reduction, however, did not translate to leukopenia since a leukocyte count below 4,900-5,100/ μ l is considered to cause leukopenia in cows (Roland, Drillich, & Iwersen, 2014). The Bovilis Nasalgen PI3 vaccinees and the negative controls recovered by day 12 post-challenge, while recovery in the rBPI3V_{cmut}F2HN2 vaccinees occurred by day 18 (Fig. 2.10C).

Platelet counts decreased across all treatment groups by day 6 post-challenge (Fig. 2.10D). However, by day 12, the rBPI3V_{cmut}F2HN2 vaccinees exhibited a higher recovery rate (from 290 K/ mm^3 to 372.7 K/ mm^3) compared to the negative controls (from 322.4 K/ mm^3 to 414.4 K/ mm^3). By day 15 post-challenge, the mean platelet count in the negative control group, compared to the vaccine treatment groups, declined to the lowest level recorded throughout the challenge phase, reaching 237.6 K/ mm^3 . This reduction, however, did not translate to thrombocytopenia, given that a Platelet count below 160-300 K/ mm^3 is considered to cause thrombocytopenia in cows (Roland et al., 2014). By day 18 post-challenge, all the vaccinees and the negative controls had recovered, with the rBPI3V_{cmut}F2HN2 vaccine treatment group recording the highest mean platelet counts (483.25 K/ mm^3) (Fig. 2.10D).

2.3.7 rBPI3V_{cmut}F2HN2 vaccinees had lower viral loads

Following challenge, the rBPI3V_{cmut}F2HN2 vaccine treatment group shed the lowest amount of virus in nasal fluids compared to the virus detected in the Bovilis Nasalgen PI3 vaccinees as well as the sham treatment controls throughout the challenge period, although this was not statistically significant (Fig. 2.11A). Notably, on day 3 post-challenge, shedding of the challenge virus was not detected in 1 out of 5 of the rBPI3V_{cmut}F2HN2 vaccinees (Fig. 2.11A). On day 6 post-challenge, peak mean shedding of the challenge virus by the rBPI3V_{cmut}F2HN2 vaccinees (detected at 1:20 dilution) was lower than the peak titers detected in the Bovilis Nasalgen PI3 vaccinees (detected at 1:45 dilution) (Fig. 2.11A). Viral shedding subsequently decreased in both groups and resolved by day 15. In contrast, the negative control group exhibited prolonged shedding of the challenge virus, with peak mean shedding occurring on day 9 post-challenge (Fig. 2.11A). On day six post-challenge, the rBPI3V_{cmut}F2HN2 vaccinees had lower viremia in blood than the Bovilis Nasalgen PI3 vaccinees and the sham negative controls (Fig. 2.11B). Compared to the Bovilis Nasalgen PI3 vaccinees and the sham controls, the rBPI3V_{cmut}F2HN2 vaccinees had lower, but insignificant, viremia in blood on day 6 (Fig. 2.11B). On day 15 post-challenge, the rBPI3V_{cmut}F2HN2 vaccinees had significantly ($p=0.0223$) lower viremia compared to the sham treatment group, but not the Bovilis Nasalgen PI3 vaccine treatment group (Fig. 2.11 C). Therefore, intranasal immunization of calves with the rBPI3V_{cmut}F2HN2 prototype vaccine protected calves from severe disease and reduced viral loads in nasal secretions and peripheral blood.

2.3.8 Gross and Histopathologic lesions

The pulmonary lesions were overall quite mild but varied. Gross lesions identified in calves from this study included: none (grossly normal lung); patchy to coalescing atelectasis (3 calves in each

of the rBPI3V_{cmut}F2HN2 and the Bovilis Nasalgen PI3 vaccine treatment groups, 2 calves in the negative control group); mild fibrinous pleuritis or fibrous pleuritis (3 calves in each of the rBPI3V_{cmut}F2HN2 vaccine treatment group and the Negative control group, 2 calves in the Bovilis Nasalgen PI3 vaccine treatment group); mild pleural or pericardial effusion (30 ml fluid in # 5651, Bovilis Nasalgen PI3 vaccine treatment group); petechial hemorrhage of epicardium (1 calf in the Bovilis Nasalgen PI3 vaccine treatment group); and pleural hemorrhage (1 calf in the Negative control group). The most common significant gross finding was mild to severe cranioventral reddened and collapsed lung (atelectasis, 10% -80% of parenchyma affected), which was present to some degree in all groups. The severity ranged from mild to severe, with calf #5669 from the Negative control group being the worst affected (Fig. 2.12).

The most common histopathologic lesions observed in all groups included poor overall alveolar aeration (atelectasis), lymphoplasmacytic and histiocytic interstitial pneumonia, neutrophilic bronchitis and bronchiolitis, lymphoplasmacytic peribronchiolitis, bronchiolitis obliterans, and bronchial or bronchiolar associated lymphoid hyperplasia. Importantly, the most severe histologic lesions were observed in calf 5669, one of the negative control calves, characterized by lymphoid hyperplasia, poorly aerated alveolar spaces with edema and alveolar macrophages, bronchiolitis obliterans characterized by partial to complete filling of bronchiolar lumens by fronds of proliferative fibrous tissue lined by airway epithelium, chronic interstitial pneumonia characterized by expansion of interstitial septa by lymphocytes and histiocytes, or proliferation of type 2 pneumocytes (Fig. 2.13).

Interestingly, tissue response and repair characterized by lymphoid hyperplasia and bronchiolitis obliterans, respectively (J. A. Ellis, 2010), were more prominent in the rBPI3V_{cmut}F2HN2 vaccine treatment group and the Bovilis Nasalgen PI3 vaccine treatment

groups. However, no significant differences in gross and histologic lesions were observed between the three groups (Fig. 2.14).

2.4 Discussion

There is a need for efficacious and broadly protective vaccines to improve the management of Bovine Parainfluenza virus infections in cattle. The primary objective of this study was to develop a BPI3V genotype-C virus backbone as an attenuated replication-competent viral vector, utilize the vector to generate attenuated recombinant BPI3Vc prototype vaccine, and evaluate its ability to safely confer protection against diverse BPI3V genotypes A-C strains in cattle. The wild-type BPI3Vc strain TVMDL 16, the BPI3Vc reference strain in the U.S., was modified to contain attenuating mutations similar to the current commercial BPI3Va-based vaccines (Fulton et al., 2017; Aurelia A. Haller et al., 2001; Aurelia A Haller, Miller, Mitiku, & Coelingh, 2000), with cloning sites for single-step insertion of transgenes of interest in the non-coding region downstream of the gene encoding the N protein, a position that allows optimal expression of transgenes while retaining the genetic stability of the negative sense RNA virus (Fig. 2.1A) (Liang et al., 2014). The modified backbone was used to generate a recombinant BPI3Vc_{mut}GFP virus (rBPI3Vc_{mut}GFP) that expressed the transgene efficiently and displayed a temperature sensitive attenuation phenotype, similar to the BPI3Va (Kansas/15626/84) vaccine strain, which has been shown to have reduced replication in the lower respiratory tract of animals (Fig. 2.1E and G) (Aurelia A. Haller et al., 2001; Aurelia A Haller et al., 2000). Live attenuated vaccines against BPI3V in cattle from back in the 1970s to date are temperature sensitive mutants (Bryson et al., 1999; Vangeel et al., 2009; Zygraich, 1979). The rBPI3Vc_{mut}GFP virus replicated well at 37°C and at a reduced rate at 39°C, but it had significantly reduced replication at 41°C *in vitro* (Fig. 2.1G).

In comparison, replication of the wild-type BPI3V_{cwt}TVML16 virus was not significantly affected across the temperature range (Fig. 2.1F). This outcome is consistent with previous findings that demonstrated utility of the BPI3V backbone as a suitable vector for *in vivo* antigen delivery in multiple species (Greenberg et al., 2005; Aurelia A Haller et al., 2003; Karron et al., 2012; Le Nouën et al., 2022; Liang et al., 2014; Ohkura et al., 2015; Pennathur et al., 2003; Tang et al., 2004). The temperature-sensitive attenuated viruses have been safely utilized as vaccine strains for mucosal immunization against PI3V infections in the United States and Europe and for development of live-vectored subunit vaccines (Aurelia A. Haller et al., 2001; Aurelia A Haller et al., 2000; Liang et al., 2014; Vangeel et al., 2009).

Although the BPI3V Fusion (F) and Hemagglutinin-Neuraminidase (HN) antigens are the major targets of protective neutralizing antibodies (Haanes et al., 1997; Karron et al., 1995; van Wyke Coelingh et al., 1990; Jiaqi Zhang et al., 2024), current commercial PI3 vaccines are based on the naturally attenuated BPI3Va strains (including SF-4 and Kansas/15626/84) (Bailly et al., 2000; Bryson et al., 1999; Fulton et al., 2017; Zygraich, 1979), which can confer protection against most, but not all, homologous strains. However, efficacy of the vaccines for protection of heterologous BPI3V genotypes B and C strains is suboptimal and generally variable (Fulton et al., 2017; Neill et al., 2015). The current commercial vaccines have been in use for many years, but they have not had a significant impact on reducing BPI3V prevalence in U.S. cattle herds, underscoring the need for improved vaccines (Fulton et al., 2017; Neill et al., 2015; Newcomer et al., 2017). In this study, novel mosaic polypeptides, F2 and HN2, separated by a 2A autocleavable motif, were designed based on consensus amino acid from all sequenced BPI3V genotypes A-C to capture determinants with potential to elicit broad protection. The mosaic F2 polypeptide sequence shares 74.1% identity to 62 F protein sequences from the BPI3V genotypes A, B, and C, while the

HN2 polypeptide sequence was 77.5% identical to 35 HN protein sequences that were available from the BPI3V genotypes A, B, and C strains (Fig. 2.2 B and E). The F2HN2 polypeptide sequences share 93.5% and 92.8% identity with the F sequence of BPI3Va SF-4 and Kansas/15626/84 vaccine strains, respectively (Fig. 2.2C); and 86.5% and 85.8% identity with the HN sequence of BPI3Va SF-4 and Kansas/15626/84 vaccine strains, respectively; (Fig. 2.2F). This approach allowed inclusion of determinants from disparate strains for broad epitope coverage to improve protective immunity against BPI3V genotypes A-C strains. Use of mosaic antigens capable of inducing broadly protective immunity has been used to generate promising vaccine candidates (Barouch et al., 2010; Broecker et al., 2019; Chang et al., 2021; Florek et al., 2017; Xuejie Liu et al., 2024; Lokhandwala et al., 2017; Mwangi, Fang, Lokhandwala, Bray, & Waghela, 2014; Sangewar et al., 2020; Jialu Zhang et al., 2024). More importantly, the F2HN2 multicistronic expression cassette can easily and rapidly be updated to include protective determinants from emerging strains (Fig. 2.2). Compared to the sequences from BPI3V genotypes A and B, the F2 and HN2 polypeptide sequences have higher homology (95% and 100%, respectively) to the F and HN sequences from genotype C strains given that there were more sequences for this genotype available (29/62 F sequences and 28/35 HN sequences, respectively). In addition, the bias was because residues from the BPI3V_{Cwt}TVML16 virus were selected if there was neither a consensus nor a dominant amino acid at a specific position following multiple sequence alignment. This strategy can be used to design a prototype vaccine that favor the predominant strain circulation at a given time period.

The F2HN2 mosaic antigens expressed by cells transfected with a plasmid construct, pCDNA-F2HN2, or cells infected with a recombinant virus, rBPI3V_{Cmut}F2HN2, generated using a codon-optimized synthetic gene encoding the F2HN2 proteins, were strongly recognized by tag-

specific and more importantly, by anti-BPI3V polyclonal antibody (Fig. 2.3). These two antigens, encoded by a multicistronic expression cassette, were expressed independently as judged by Western Blot analysis demonstrating that the 2A autocleavable motif, which separate F2 from HN2, worked as expected (Fig. 2.3E) (Ryan et al., 1991). More importantly, the ectodomains of the mosaic antigens were displayed on the surface of rBPI3V_{cmut}F2HN2-infected cells, for optimal recognition of linear as well as conformational epitopes by B-cells (Fig. 2.4) (Liang et al., 2016). Insertion of the transgene encoding GFP (~0.8 kb) or F2HN2 (~3.6 kb) between the N and P in the BPI3V_{cmut} backbone had minimal effect on the replication of the resultant recombinant viruses, which is consistent with previous demonstrations that the BPI3V backbone can successfully be used to generate stable recombinant viruses expressing transgenes of interest (Fig. 2.5) (Bukreyev et al., 2004; Gomez et al., 2009; Le Nouën et al., 2022; Liang et al., 2014; Liang et al., 2016; Xueqiao Liu et al., 2021; Tang et al., 2004). The observed lower titers of the rBPI3V_{cmut}GFP and the rBPI3V_{cmut}F2HN2 viruses in the first three days after infection of MDBK cells, suggest an attenuated less vigorous phenotype, similar to attenuated phenotypes observed with RNA virus-vectored vaccines (Fig. 2.5) (Bukreyev, Skiadopoulos, Murphy, & Collins, 2006; Aurelia A Haller et al., 2000). Additionally, the BPI3V_{cmut} backbone and the gene encoding the F2HN2 polypeptides remained relatively stable *in vitro* after nine passages, with only two point mutations in the HN gene (K254R) and L gene (S2220Y, S2221L) (Table 2.1), suggesting that the vector backbone is relatively stable and maintains transgene expression over several passages, which are attractive traits for a vaccine vector (Bukreyev et al., 2006; Phan et al., 2017). Empirical data from *in vivo* studies is required to assess the risk of potential reversion to virulence, but no such issues have been reported so far for the current BPI3Va commercial vaccines.

The rBPI3V_{cmut}F2HN2 prototype vaccine was well tolerated by five-month-old calves following intranasal immunization and the effect on body temperature and weight gain was similar to that observed in calves immunized with the commercial vaccine (Fig. 2.6). The prototype vaccine primed serum and mucosal IgG responses that strongly recognized representative wild-type BPI3V genotypes A-C strains (Fig. 2.7). Notably, compared to the IgG responses induced by the commercial Bovilis Nasalgen[®] IP (Merck) vaccine, the IgG responses elicited by the rBPI3V_{cmut}F2HN2 prototype vaccine were significantly amplified in sera and in nasal fluids after the first, but not the second booster dose, suggesting that a single booster dose could be sufficient to stimulate robust humoral responses (Fig. 2.7). More importantly, the antibodies primed and expanded by the rBPI3V_{cmut}F2HN2 prototype vaccine neutralized representative wild-type BPI3V genotypes A-C strains with exceptionally high virus neutralization titers (VNTs) that were significantly ($p < 0.0001$) higher than VNTs elicited by the commercial Bovilis Nasalgen vaccine (Fig. 2.8), suggesting that the prototype vaccine has potential to confer better protection. This outcome is significant given that there is insufficient data to show the neutralization breadth and potency of current BPI3V vaccines against emergent heterologous BPI3V strains in the U.S. (Fulton et al., 2016; Fulton et al., 2017; Masic et al., 2020). In addition, existing reference serum has low neutralizing titers against heterologous BPI3V strains (Neill et al., 2015). Specifically, the APHIS 475 BDV 0601 reference serum (BPI3Va SF-4 strain) has less than 1:32 titer against BPI3Va-c strains (Neill et al., 2015) and sera from field infection studies have been reported to have VNTs of up to 1: 512 (Gaeta et al., 2017). Notably, the VNTs elicited by the rBPI3V_{cmut}F2HN2 prototype vaccine (Fig. 2.8) were much higher compared to VNTs elicited by a baculovirus vectored-HN vaccine (VNT at 1:96), adenovirus vectored-F (VNT at 1:256), and adenovirus vectored-FHN (VNT at 1:426) experimental vaccines (Jiaqi Zhang et al., 2024; Zhang

et al., 2023). Interestingly, the robust BPI3Va-c-specific VNTs elicited by the rBPI3V_{cmut}F2HN2 prototype vaccine were mainly driven by the mosaic F2HN2 antigens since the BPI3Va-c-specific VNTs induced by the vector control construct expressing an irrelevant antigen, rBPI3V_{cmut}TMSP virus, were significantly lower and similar to the VNTs elicited by the commercial vaccine, which confirmed that the vector backbone also elicited VNTs (Fig. 2.9). This is likely due to higher expression of the gene encoding the mosaic F2HN2 antigen since it is located closer to the promoter and it could also likely be due to codon-optimization.

Intranasal immunization of calves at an early age can prime immune responses that can contribute to an early onset of protection in calves as young as one week old, even in the presence of maternally derived antibodies (J. A. Ellis, 2010; Metcalfe et al., 2020; P Nuijten et al., 2019). Immunization of calves with the rBPI3V_{cmut}F2HN2 prototype vaccine induced strong mucosal and serum antibody responses, which would be desirable for blocking host infection through the mucosal infection portals and or reducing disease severity once infection occurs (J. A. Ellis, 2010; Frank & Marshall, 1971; R. Marshall & Frank, 1971). BPI3V-specific neutralizing titers of 1:32 are generally deemed protective (J. A. Ellis, 2010; Frank & Marshall, 1971; R. Marshall & Frank, 1971; Masic et al., 2020). The duration of immunity conferred by a single dose of bivalent Bovilis Nasalgen® IP used in this study has not been established, a similar pentavalent product, Bovilis Nasalgen® 3-PMH, only confers protection for up to 3 months (Bulletin, 2020). The optimal dose and duration of immunity elicited by the rBPI3V_{cmut}F2HN2 prototype vaccine will need to be empirically determined.

Challenge resulted in mild clinical signs, with a majority of the calves maintaining a temperature below 103.1°F. The normal rectal temperature in healthy calves ranges between 101-103°F. A temperature above 103.5°F is considered a sign of infection (Alvarez et al., 2023). Similar

to previous studies, there was no significant difference in pyrexia between vaccinated and non-vaccinated calves (Fig. 2.10) (Flynn et al., 2024; van Rooij et al., 2023). Although all calves exhibited steady weight gain throughout the challenge phase, the rate of weight gain was much lower in negative control calves compared to their vaccinated counterparts (Fig. 2.10). Unexpectedly, calves in all the treatment and control groups experienced a decrease in leukocyte and platelet counts, however, calves immunized with the rBPI3V_{cmut}F2HN2 vaccine exhibited a higher recovery rate compared to the negative control group (Fig. 2.10). Notably, the decreases observed in this study did not fall below the thresholds typically associated with leukopenia (4,900–5,100/ μ l) or thrombocytopenia (160–300 K/ mm^3) in cows (Roland et al., 2014). There is limited information on the association between BPI3V infection in cattle and the development of leukopenia and thrombocytopenia. Therefore, it is possible that other confounding factors could have contributed to these observations. Following challenge, the calves immunized with the rBPI3V_{cmut}F2HN2 prototype vaccine shed the lowest amount of virus in nasal swabs and had lower viremia in blood throughout the challenge phase (Fig. 2.11). The negative control calves shed the virus longer with peak shedding occurring on day 9, while viral loads in blood in the rBPI3V_{cmut}F2HN2-vaccinated calves were significantly lower than those in the sham-treated negative control group (Fig. 2.11). This demonstrated that intranasal immunization of calves with the rBPI3V_{cmut}F2HN2 prototype vaccine protected calves from severe disease, and reduced viral loads in blood and nasal fluid, which is consistent with other intranasally delivered BPI3V vaccines (R. Marshall & Frank, 1971; Metcalfe et al., 2020; P Nuijten et al., 2019; van Rooij et al., 2023).

Gross and histopathologic lung lesions observed in challenged calves were consistent with acute BPI3V infection (J. Ellis et al., 2020; J. A. Ellis, 2010). Mild gross lung lesions, particularly atelectasis, in the rBPI3V_{cmut}F2HN2 vaccinated calves were observed, while moderate and severe

lesions were observed in the Bovilis Nasalgen and negative control groups, respectively (Fig. 2.12), which was similar to observations made in previous studies (van Rooij et al., 2023). Histopathologic assessments confirmed the presence of BPI3V lesions including interstitial pneumonia, neutrophilic bronchiolitis, and lymphoplasmacytic peribronchiolitis in all treatment groups. Although there were no significant differences in gross and histologic lesions between the groups, slightly elevated disease scores were recorded in the negative control group, particularly calf 5669, which exhibited severe interstitial pneumonia (Fig. 2.13), underscoring the severity of disease in non-vaccinated feedlot cows. The observed higher scores for lymphoid hyperplasia in the rBPI3V_{cmut}F2HN2 vaccinees and bronchiolitis obliterans in both vaccinated groups could be associated with mechanisms initiating repair of the airway epithelium, which occurs approximately 14 days post-infection, and which is characterized by hyperplasia of the airway and alveolar epithelium and in some cases organized fibroplasia and neovascularization of airway connective tissue wall (bronchiolitis obliterans) following bronchiolar injury (J. Ellis et al., 2020; J. A. Ellis, 2010).

The data presented here provide a platform for improving the management of bovine parainfluenza viruses. Unlike existing MLVs, subunit vaccines have the advantage of being amenable to covering strains of interest with changing genetic landscapes. Recent studies show the efficacy of other subunit vaccines in protecting against BPI3V infections. Two studies indicated that a replication-incompetent adenovirus vectored BPI3V fusion, or fusion and hemagglutinin-neuraminidase vaccines, elicited BPI3V-specific viral neutralizing antibodies, splenic CD3⁺/CD8⁺ T cell proliferation, and production of inflammatory and anti-inflammatory IFN- γ /IL-4 cytokines. (Jiaqi Zhang et al., 2024; Zhang et al., 2023). In a separate immunogenicity study, an intranasal BPI3V whole virus antigen encapsulated in nanoparticles elicited stronger mucosal IgA than the

commercial intranasal vaccine (Mansoor et al., 2015). Additionally, another study found that a baculovirus vectored hemagglutinin neuraminidase vaccine elicited neutralizing antibodies that protected calves following challenge (Haanes et al., 1997). It is worth noting that given the VN titers in all these studies were less than 1:426, VN titers presented in the current study seem to be more robust and broadly protective against BPI3Va-c genotypes (1:8192/1:4096) compared to previously reported subunit vaccine candidates, or even other commercially available MLVs from recent studies conducted in North America and Europe (Flynn et al., 2024; Masic et al., 2020; Metcalfe et al., 2020; Piet Nuijten et al., 2022). In Europe in particular, these recent studies have optimized the MLV vaccines, showing that intranasal immunization of young calves is critical in inducing mucosal immunity, which contributes to an early onset of protection as young as right after birth (van Rooij et al., 2023) or at one week old (P Nuijten et al., 2019), even in the presence of maternal antibodies (Metcalfe et al., 2020). Such safe efficacious intranasal vaccines are yet to be tested in newborn calves in North America, and our rBPI3V_{cmut}F2HN2 prototype vaccine can be a good candidate for further empirical testing in neonates.

In conclusion, results from this study showed that a BPI3Vc vector with targeted attenuating mutations was successfully developed with unique cloning sites for generation of recombinant live-vectored prototype vaccines that can easily be refined to fit future needs for improved broad protection against diverse BPI3V strains. In addition, a multicistronic expression cassette encoding novel mosaic F2HN2 polypeptides designed using protein sequences from BPI3V genotypes A-C strains was generated and the rBPI3V_{cmut}F2HN2 prototype vaccine developed using the cassette was shown to display the antigens on the surface of infected cells for optimal B cell recognition. More importantly, intranasal immunization of calves with the rBPI3V_{cmut}F2HN2 prototype vaccine elicited potent broadly neutralizing antibodies that reduced

shedding of wild-type virus by vaccinees following challenge. Notably, it was shown that the mosaic F2HN2 antigens were responsible for driving the potent neutralizing antibodies. The vaccine platform opens up opportunities for tailormade contemporary vaccines not only for BPI3V, but for other pathogens, such as BVDV, which are responsible for causing BRD.

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Ethics Statement

Animal study was conducted in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University Institutional Animal Care and Use Committees (IACUC registration # 4825) and Institutional Biosafety Committee (IBC registration # 1684).

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Disclosures

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Table 2.1A. Sequencing rBPI3V_{cmut}F2HN2 viral RNA at Passage 2

<i>Gene</i>	Identity Percentage	Read Coverage	Genomic Coverage
<i>N</i>	100	18704.24512	99.93902439
<i>F2HN2</i>	100	11271.62067	100
<i>P</i>	100	4944.433981	99.95017439
<i>M</i>	100	2833.282197	99.81060606
<i>FHN</i>	100	2917.462546	100
<i>L</i>	99.9852833	942.4726841	99.94721562

Table 2.1B. Sequencing rBPI3V_{cmut}F2HN2 viral RNA at Passage level 9

<i>Gene</i>	Identity Percentage	Read Coverage	Genomic Coverage
<i>N</i>	100	1553.32287	99.85052317
<i>F2HN2</i>	100	9417.81721	100
<i>P</i>	100	898.5170455	99.81060606
<i>M</i>	100	66520.04268	100
<i>FHN</i>	99.97360781	302.1126946	99.94721562
<i>L</i>	99.94114185	361.4809419	99.76453274

Table 2.2. BPI3V Calf Immunization protocol

Group	Calf ID	Immunogen (IN)	Prime-boost dose per calf
1. rBPI3V_{cmut}F2HN2	5661		
	5662	rBPI3V _{cmut} F2HN2 virus	5.4 x 10 ⁸ TCID ₅₀ , 2.5 x 10 ¹⁰
	5664	Live vectored vaccine	TCID ₅₀ , 4.4 x 10 ¹⁰ TCID ₅₀
	5697		
	5701		
2. Bovilis Nasalgen IP (Intervet/Merck)	5651		
	5660	<i>Commercial MLV cocktail:</i>	
	5672	Bovine Rhinotracheitis	2 ml, 2 ml, 2ml
	5709	Parainfluenza 3	
	5713		
3. Sham (DMEM)	5652		
	5654	DMEM	2 ml, 2 ml, 2ml
	5669		
	5676		
	5726		

(IN) = Intranasal route of immunization

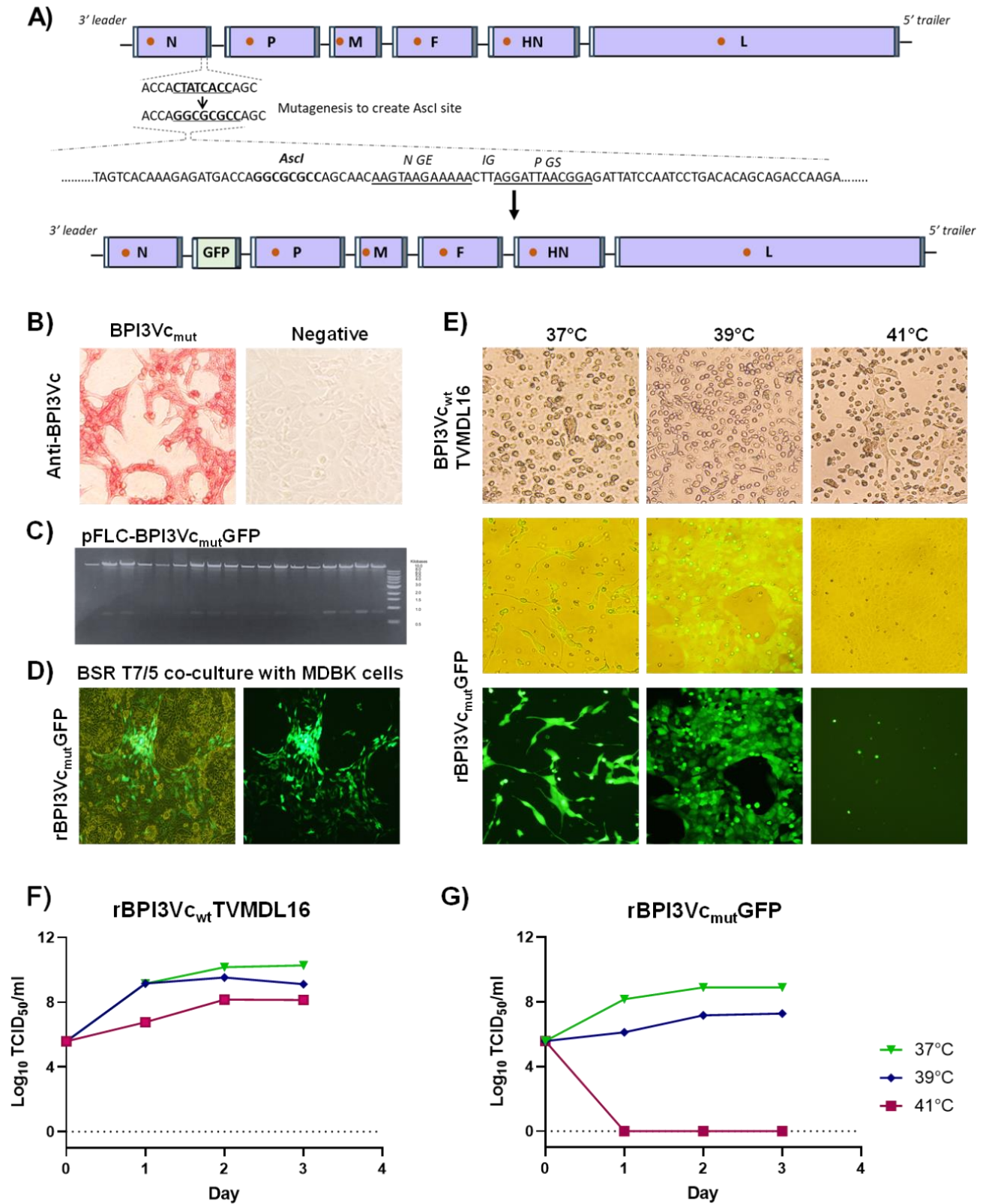
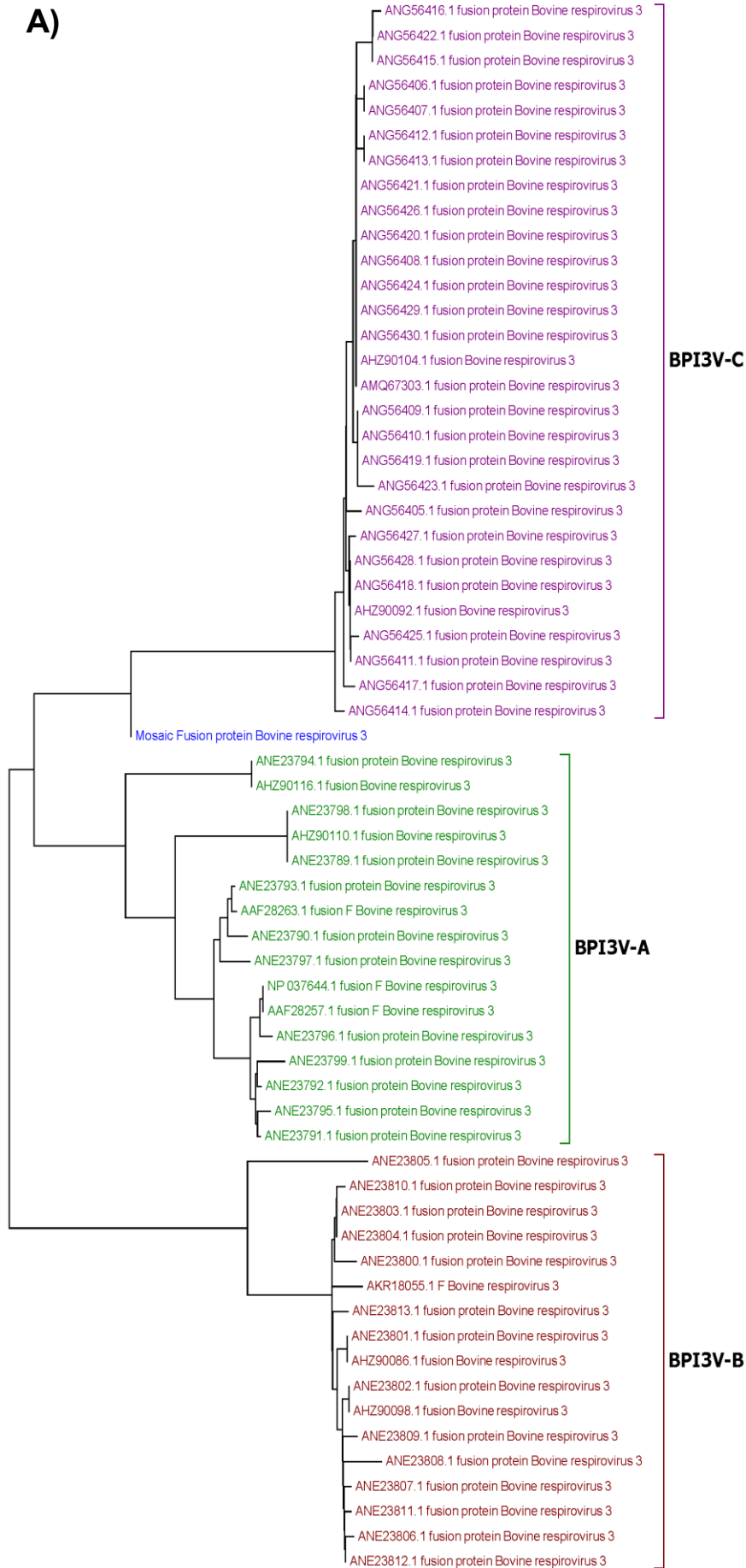


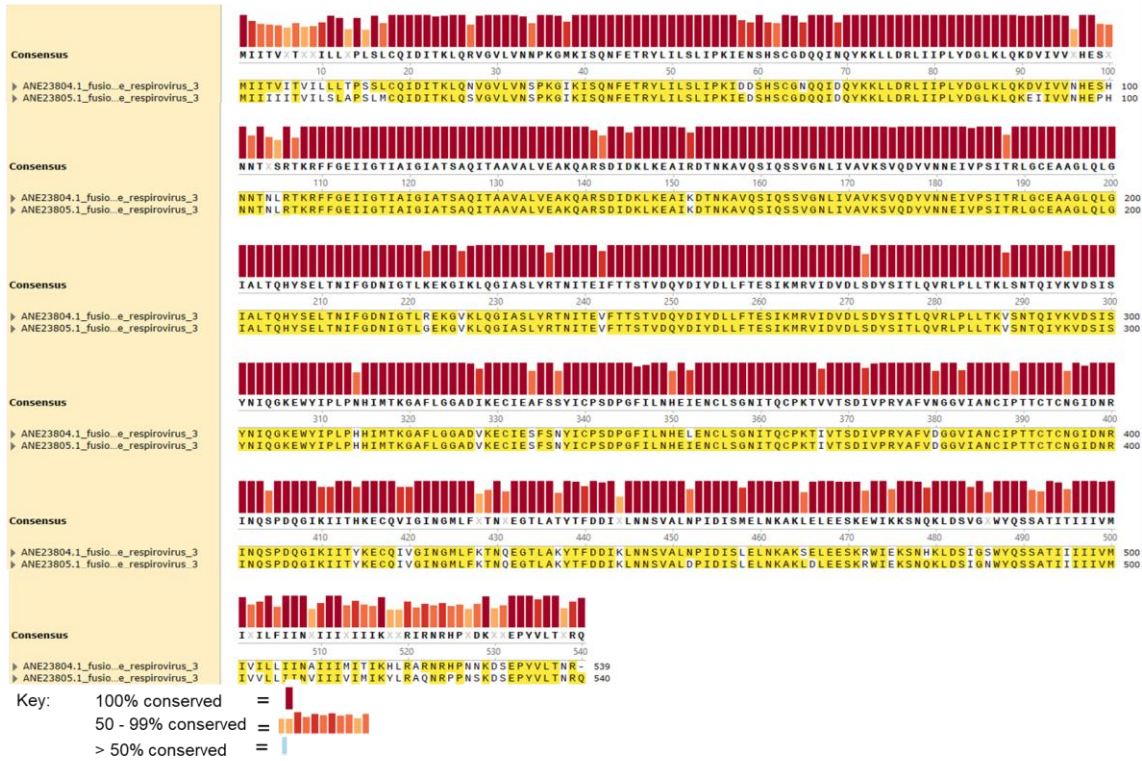
Figure 2.1. Attenuated BPI3Vc expression constructs and temperature sensitivity.

A) Design of the BPI3Vc construct encoding GFP. Transgenes were cloned, in-frame, between the genes encoding N and P and are flanked by gene start (white bar) and gene end (grey bar) transcription signals, respectively. The genes encoding N and P are separated by an intergenic trinucleotide sequence (CTT), for expression as separate mRNA; **B)** Assembled BPI3Vc_{mut} virus was authenticated by probing infected MDBK cells using anti-BPI3Vc polyclonal antibody; **C)** AscI restriction enzyme digestion of the pFLCBPI3Vc_{mut}GFP plasmid construct releasing the 15.5kb BPI3Vc vector and 0.8 kb GFP insert; **D)** Rescued recombinant BPI3Vc_{mut}GFP virus in transfected BSR T7/5 cells co-cultured with MDBK cells (left: brightfield view of invaginated BSR T7/5 cells and right: darkfield fluorescent view of infected MDBK cells); and **E - G)** Recombinant BPI3Vc_{mut}GFP virus, but not BPI3Vc_{wt}TVMDL 16 virus, is temperature sensitive. **E)** MDBK cells infected with BPI3Vc_{wt}TVMDL16 or rBPI3Vc_{mut}GFP virus incubated at 37°C, 39°C or 41°C. GFP expression and extent of infected-monolayer CPE for brightfield view (top) and darkfield fluorescent view (bottom). **(F-G)** Viral TCID₅₀ titers up to three days post-infection.

A)



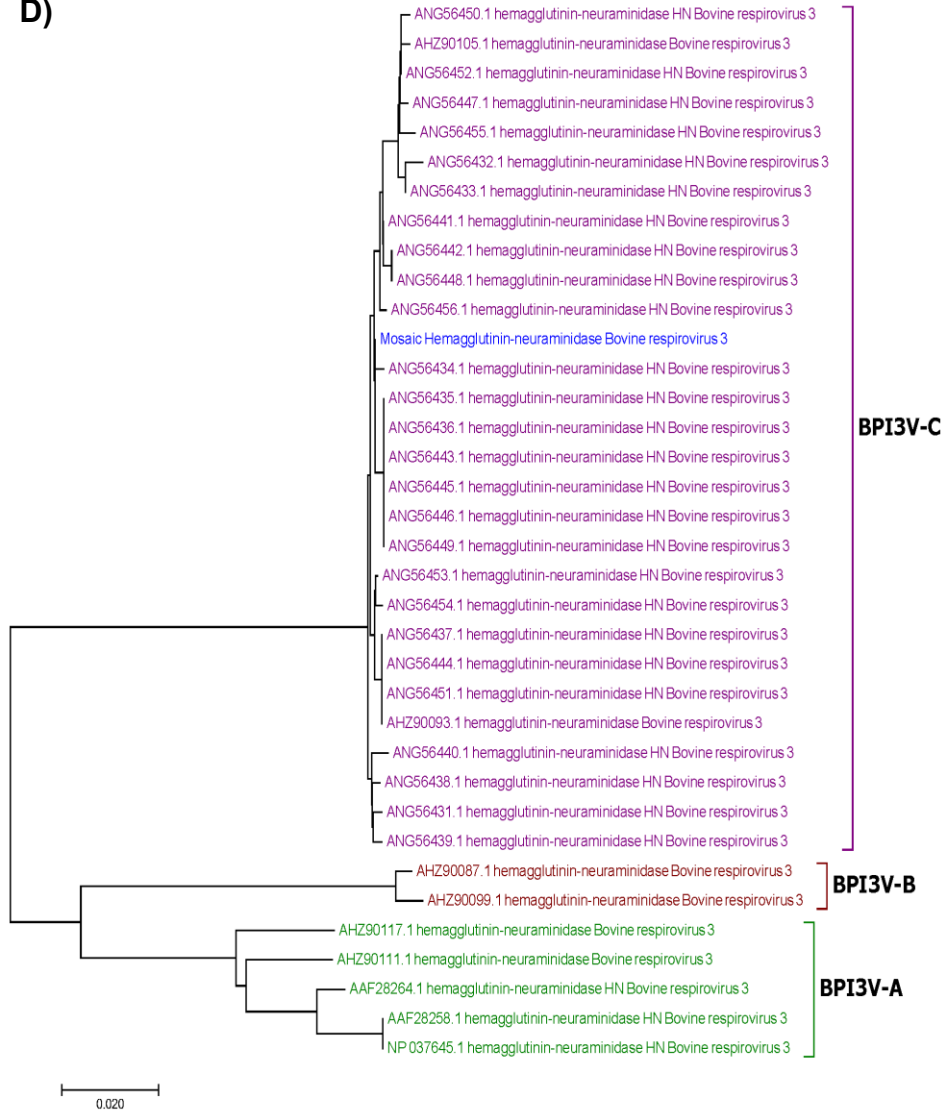
B) Mosaic Fusion [F2] sequence



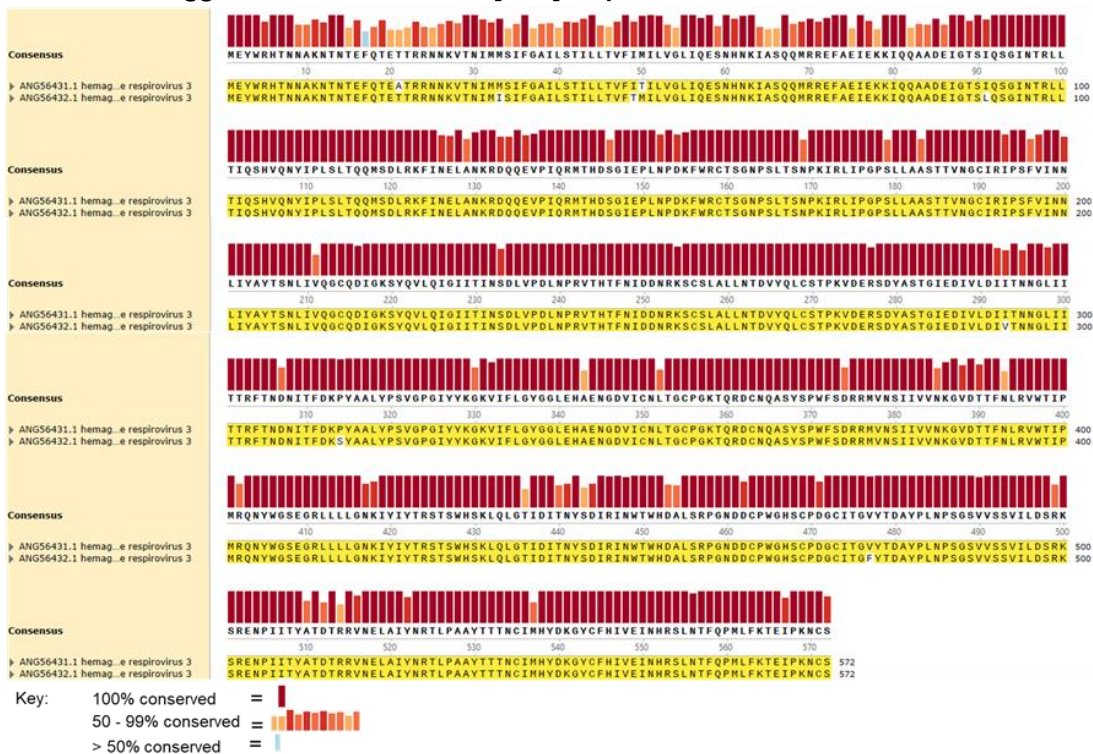
C) Kansas/15626/84 F vs Mosaic F2 sequence

AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	M	T	I	T	T	I	A	L	L	P	S	F	C	Q	I	D	I	T	K	L	R	V	G	V	L	N	N	P	K	G	M	K	I	S	Q	N	F	E	T	R	Y	L	I	L	S	L	P	K	I	E	N	S	H	S	C	G	D	Q	Q	I	N	Q	Y	K	K	L	L	D	R	L	I	I							
AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	P	L	Y	D	G	L	K	L	Q	K	D	V	I	V	S	H	E	T	H	N	N	T	N	S	R	T	K	R	F	F	G	E	I	I	G	T	I	A	I	G	I	A	T	S	A	Q	I	T	A	A	V	A	L	V	E	A	K	Q	A	X	S	D	I	E	K	L	K	E	A	I	R	D	T	N	K	A	V	Q	S
AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	I	Q	S	S	V	G	N	L	I	V	A	V	K	S	V	Q	D	Y	V	N	N	E	I	P	S	I	T	R	L	G	C	E	A	A	G	L	Q	L	G	I	A	L	T	Q	H	Y	S	E	L	T	N	I	F	G	D	N	I	G	T	L	K	E	K	G	I	K	L	Q	G	I	A	S	L	Y	R	T	N	I	T
AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	E	I	F	T	T	S	T	V	D	Q	Y	D	I	Y	D	L	L	F	T	E	S	I	K	M	R	V	I	D	V	D	L	S	D	Y	S	I	T	L	Q	V	R	L	P	L	T	K	L	S	N	T	Q	I	Y	K	V	D	S	I	S	Y	N	I	Q	G	K	E	W	Y	I	P	L	P	N	H	I	M	T	K	G
AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	A	F	L	G	G	A	D	I	K	E	C	I	E	A	F	S	S	Y	I	C	P	S	D	P	G	V	I	L	N	H	E	I	N	C	L	S	G	N	I	T	Q	C	P	K	T	V	T	S	D	V	P	R	Y	A	F	V	N	G	G	I	A	N	C	I	P	T	T	C	T	C	N	G	I	D	N	R			
AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	I	N	Q	S	P	D	Q	G	I	K	I	I	T	H	K	E	C	Q	V	I	G	I	N	G	L	F	T	N	R	E	G	T	L	A	T	Y	T	F	D	D	I	V	L	N	N	S	V	A	L	N	P	I	D	I	S	M	E	L	N	K	A	K	L	E	L	E	S	K	E	W	I	K	K	S	N	Q	K		
AAF28257.1 fusion F Respirovirus bovis Mosaic Fusion (F2)	L	D	S	V	G	S	W	Y	Q	S	S	A	T	I	T	I	I	V	M	I	I	L	V	I	N	I	T	I	V	V	I	I	K	F	H	R	I	Q	G	K	D	O	N	D	K	N	S	E	P	Y	L	T	N	R	Q																								

D)



E)



F)

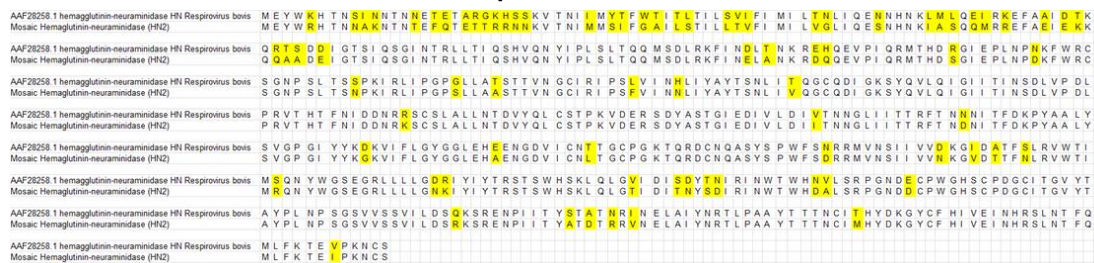


Figure 2.2. Design of mosaic Fusion (F2) and Hemagglutinin-Neuraminidase (HN2) antigens.

Clustering of the consensus F2 sequence (**A**) and HN2 sequence (**D**) (shown in blue) in relation to sixty-two BPI3V genotype A-C F and thirty-five HN polypeptide sequences, respectively. Conservation levels of mosaic F2 (**B**) and HN2 (**E**) consensus sequences. Homology between the BPI3V SF-4 and Kansas/15626/84 vaccine strains F and mosaic F2 (**C**); HN and mosaic HN2 (**D**) sequences, respectively.

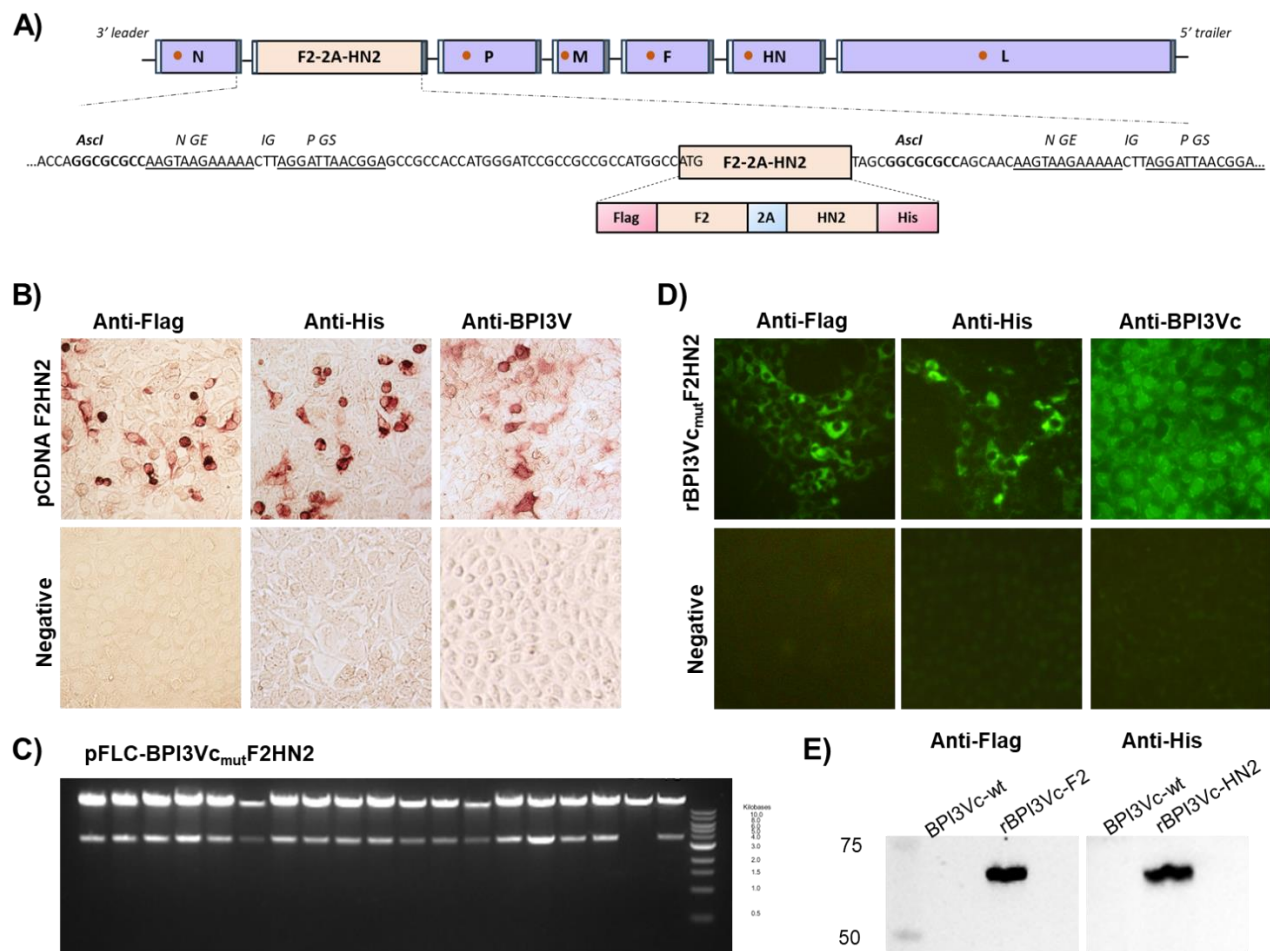


Figure 2.3. Attenuated BPI3Vc construct expressing F2HN2.

A) Design of the BPI3Vc construct encoding F2-HN2 genes; **B)** Expression of pCDNA-encoded F2HN2 mosaic proteins was validated using anti-Flag mAb (to detect F2), or anti-His mAb (to detect HN2), whereas authenticity of the F2HN2 antigens was confirmed by using anti-BPI3Vc pAb; **C)** Asci restriction enzyme digestion of the pFLCBPI3Vc_{mut}F2HN2 construct; **D)** Protein expression by MDBK cells infected with the rescued recombinant BPI3Vc_{mut}F2HN2 virus was validated using the same probes as above; and **E)** Expression of the individual F2 and HN2 antigens by the multicistronic expression cassette was confirmed by Western Blot analysis using the anti-Flag and the anti-His mAbs. Wild-type BPI3Vc TVMDL 16 virus served as the negative control.

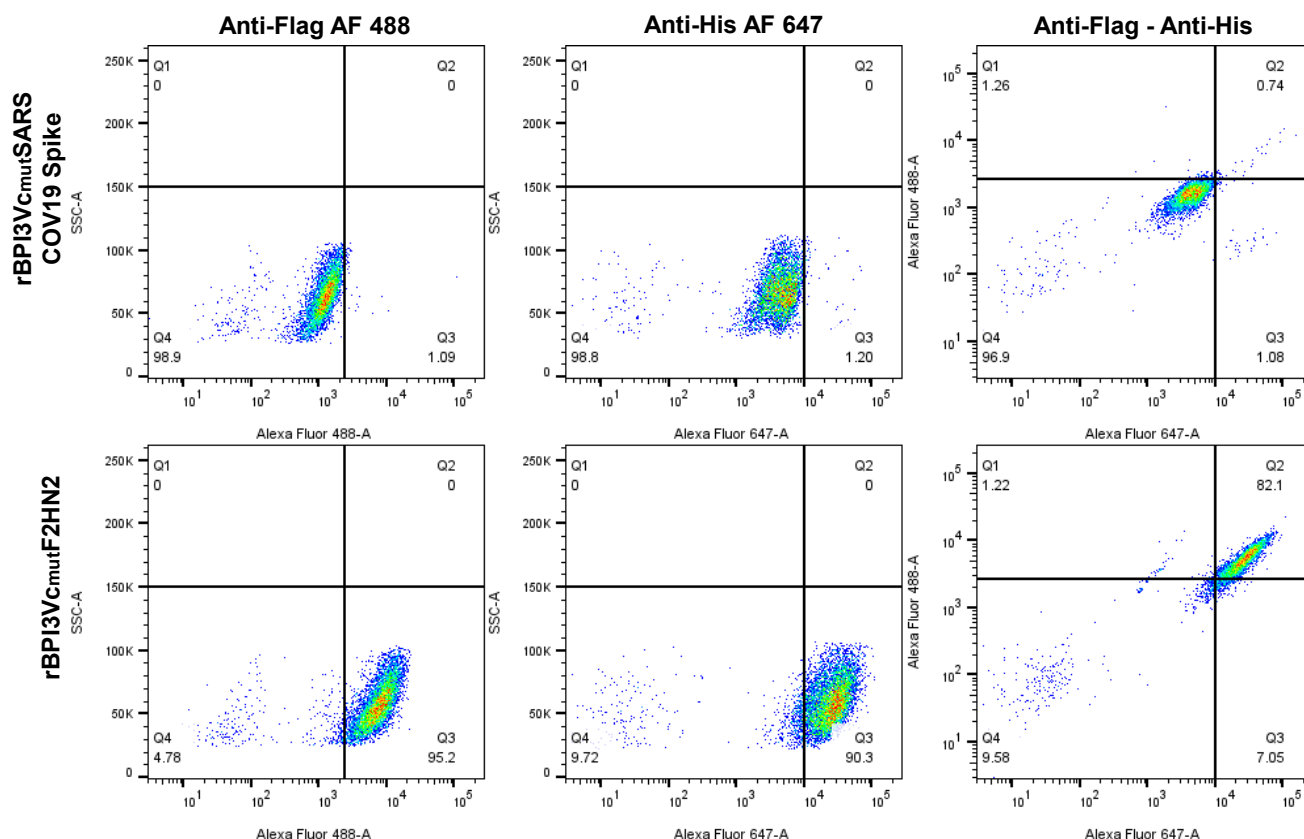


Figure 2.4. Surface display of transgene product.

Surface display of F2 on the surface of MDBK cells infected with the rescued rBPI3VcmutF2HN2 virus was validated using anti-Flag AF 488 mAb (to detect F2) by Flow cytometric analysis. A cognate irrelevant virus, rBPI3VcmutSpike virus, served as the negative control.

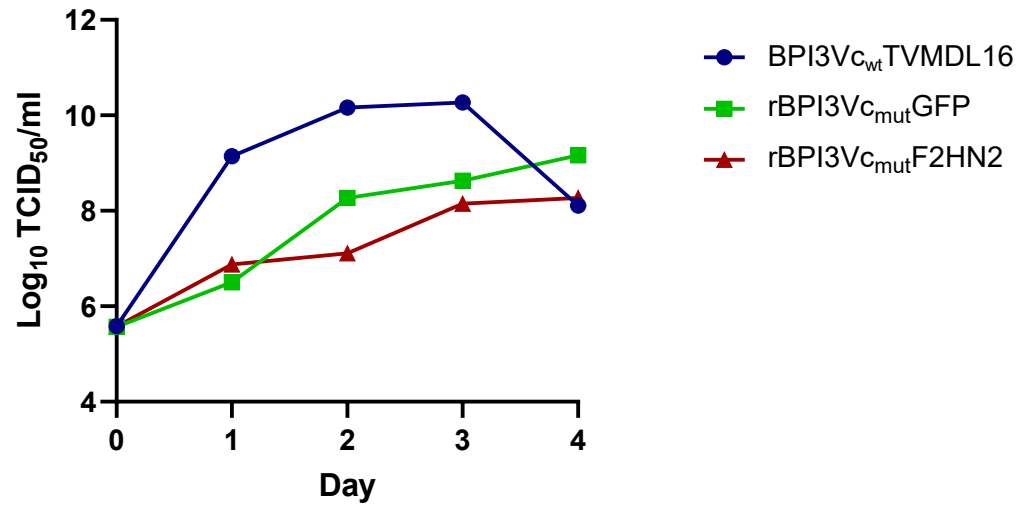


Figure 2.5. Characterization of the rBPI3Vc_{mut}F2HN2 virus.

Multicycle replication growth curve of the BPI3Vc_{wt}TVMDL16 wild-type virus, rescued rBPI3Vc_{mut}GFP, and rBPI3Vc_{mut}F2HN2 viruses in MDBK cells infected at an MOI of 1 and incubated at 37°C for four days. Virus aliquots were sampled every 24 hours and quantified by TCID₅₀ virus titration.

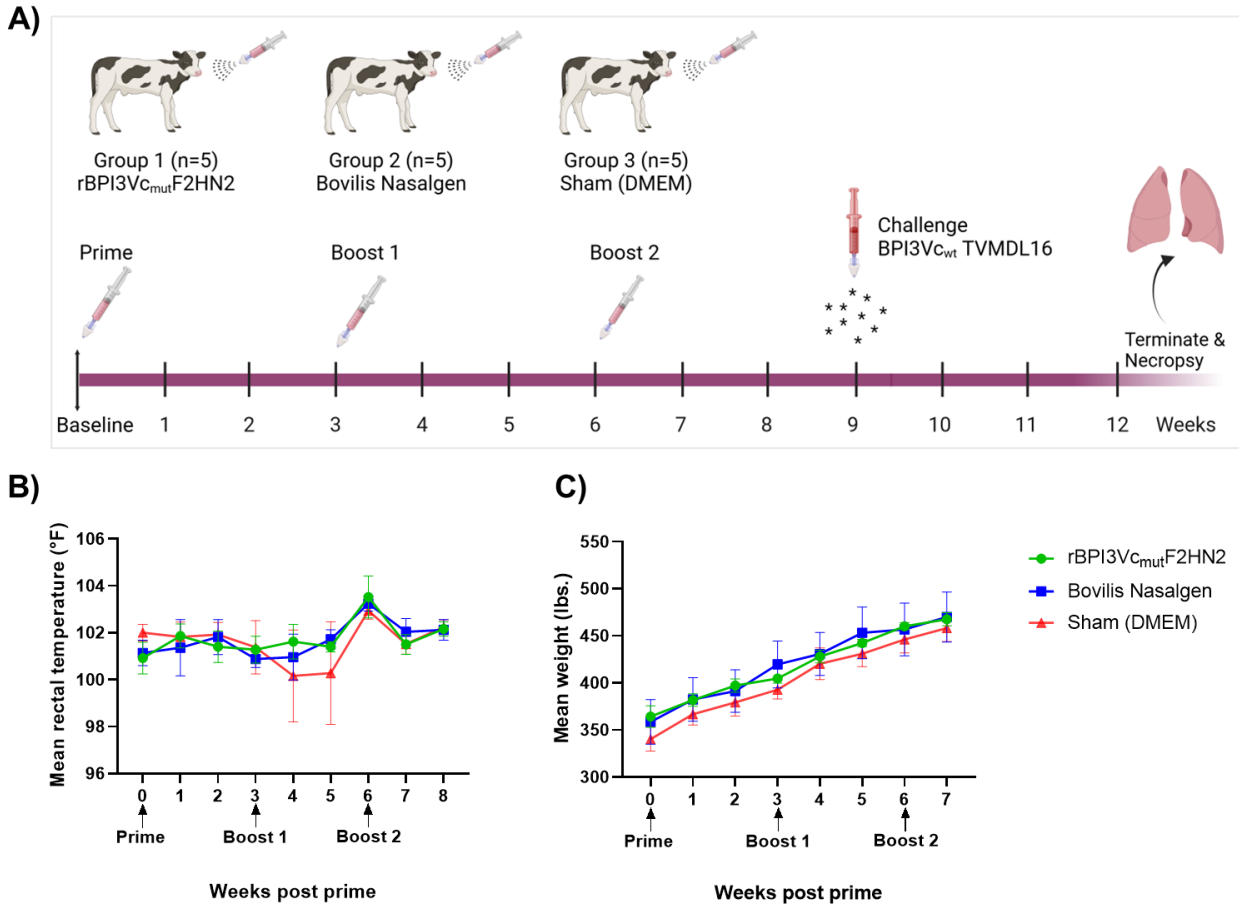


Figure 2.6. Animal Study.

(A) Immunization design and timeline. Calves were assigned to three treatment groups: 1) rBPI3Vc_{mut}F2HN2 vaccine; 2) Commercial Bovilis Nasalgen vaccine; and 3) Sham-treated with DMEM media. Following one week acclimation, the calves received intranasal instillation of the priming dose followed by booster doses three weeks apart. All calves were challenged nine weeks post-priming by intranasal administration of the wild-type BPI3Vc TVMDL16 virus and the study was terminated 13 wks after initiation of the study. Sampling for whole blood (for PBMCs and sera), and nasal swabs was done weekly; (B) Mean rectal temperatures; and (C) mean weight gain in all three groups were measured weekly.

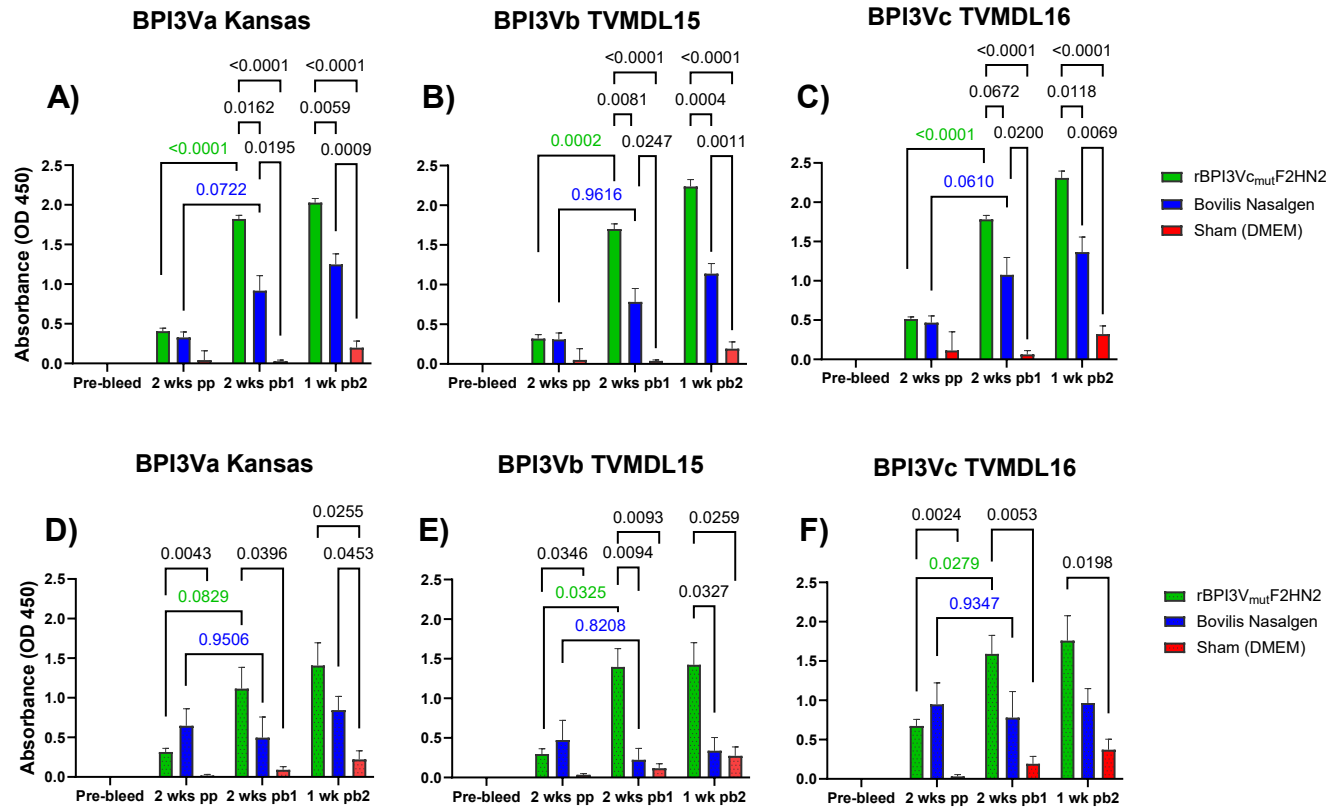


Figure 2.7. Antibody response.

Recognition of inactivated wild-type BPI3Va, b, and c virus by IgGs in sera (A-C) or nasal fluids (D-F) was evaluated by Indirect ELISA using samples collected before immunization (Pre-bleed), at two weeks post-priming (2 wks pp), two weeks post-boost 1 (2 wks pb1) and one week post-boost 2 (1 wk pb2) from all the treatment groups. Bars represent group means and the statistical differences in group means are shown.

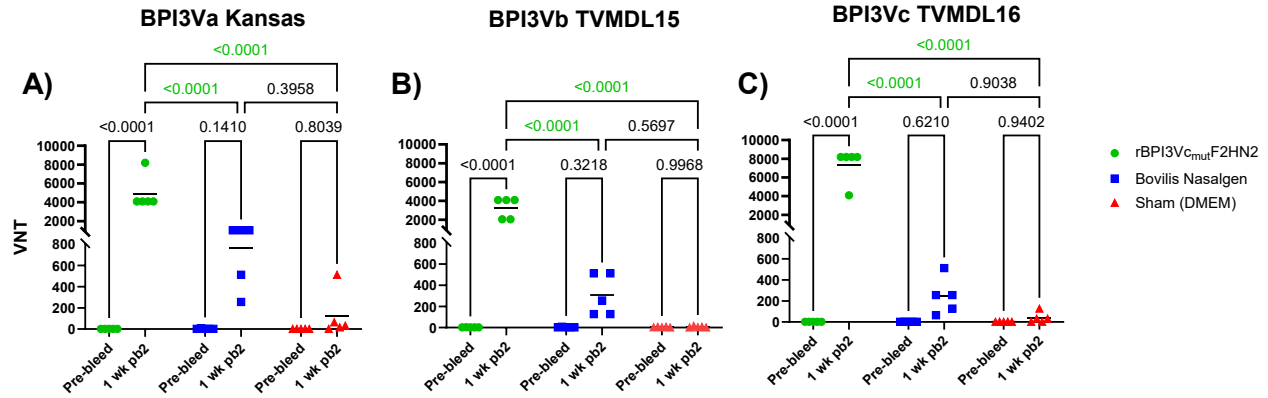


Figure 2.8. Virus neutralization.

Titers of virus neutralization antibodies in the sera from rBPI3Vc_{mut}F2HN2 vaccinees, Bovilis Nasalgen positive controls, and the sham treatment group were determined by neutralization assay against representative BPI3Va, b and c strains using sera from blood samples collected at one-week post-boost-2. Sera from cognate pre-bleed served as the baseline. Bars represent mean group titers.

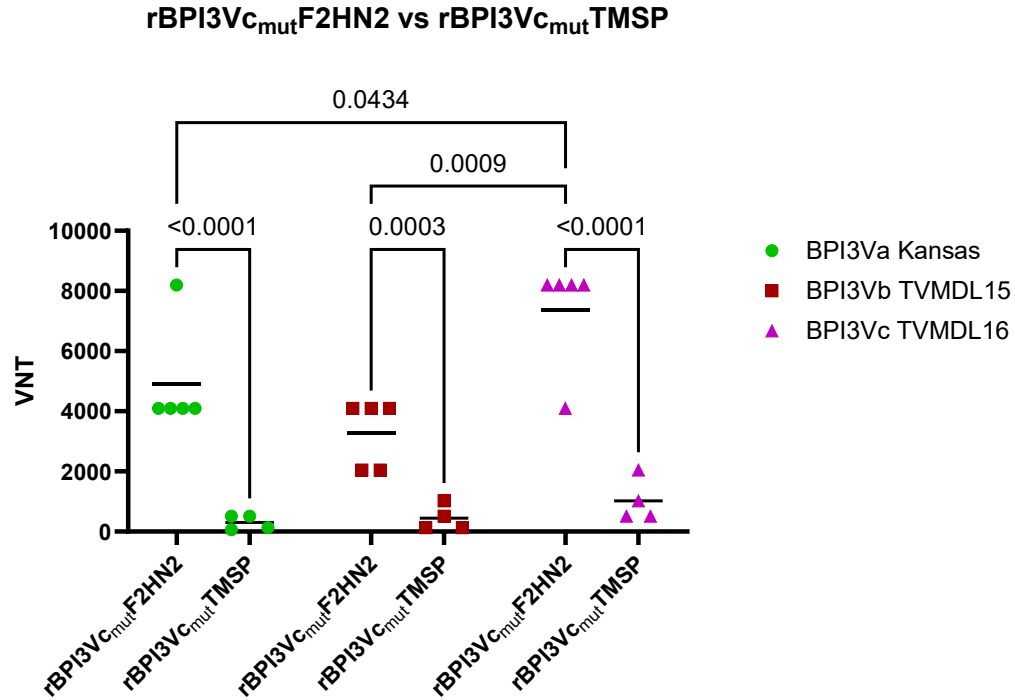


Figure 2.9. The mosaic F2HN2 antigens were responsible for the robust broadly neutralizing antibodies.

Virus neutralization assays were used to determine BPI3Va-, b- and c-specific neutralization titers in sera from blood collected from the rBPI3V_{Cmut}F2HN2 vaccinees at ten days post-boost-2 and compared to titers in sera from cattle immunized with the control rBPI3V_{Cmut}TMSP virus construct. Mean group titers are represented by bars.

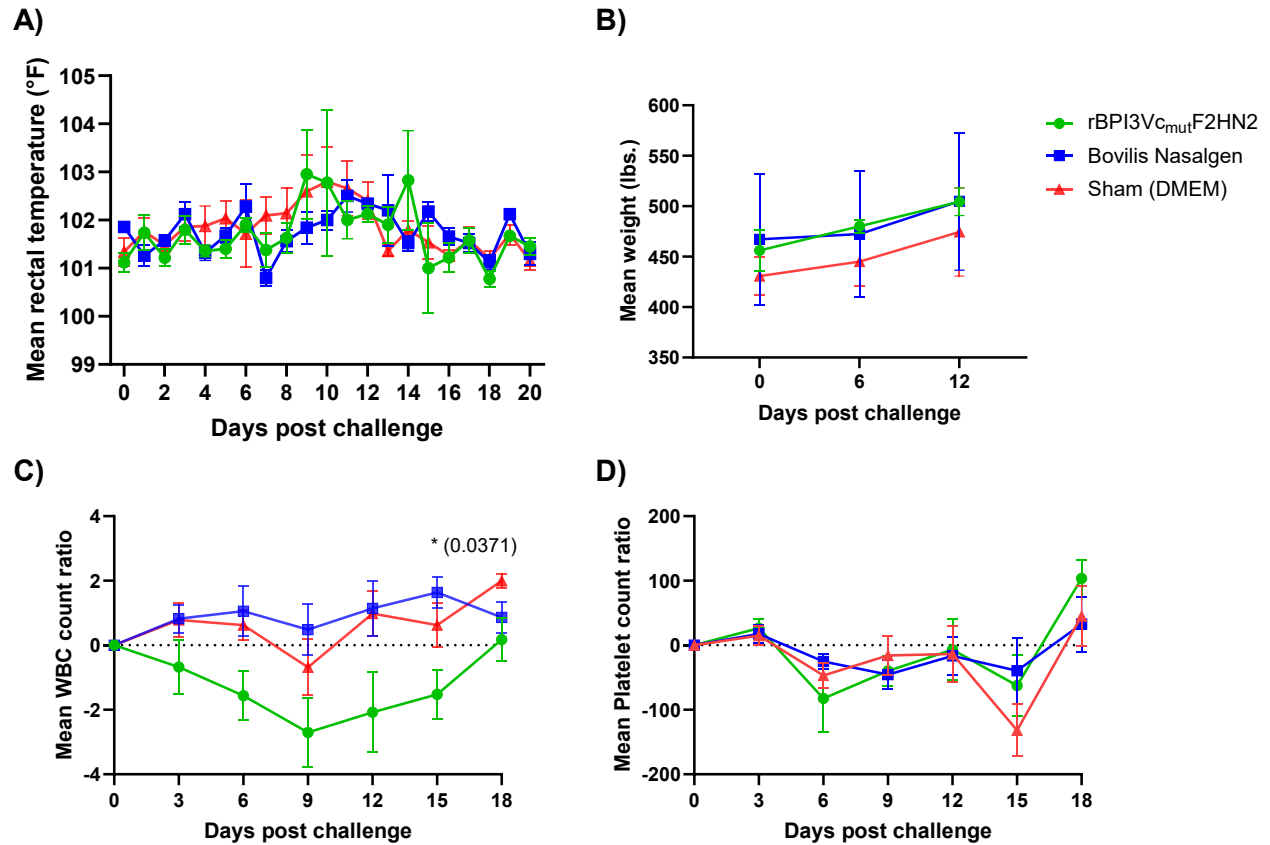


Figure 2.10. Clinical outcomes post-challenge.

A) Mean rectal temperature change for each group recorded every day post-challenge is plotted; **B)** mean gain in body weight for each group recorded on the day of challenge, six- and twelve-days post-challenge is plotted; **C)** Mean change in white blood cell; and **(D)** change in platelet count change ratios for each group quantified on every third day post-challenge is plotted.

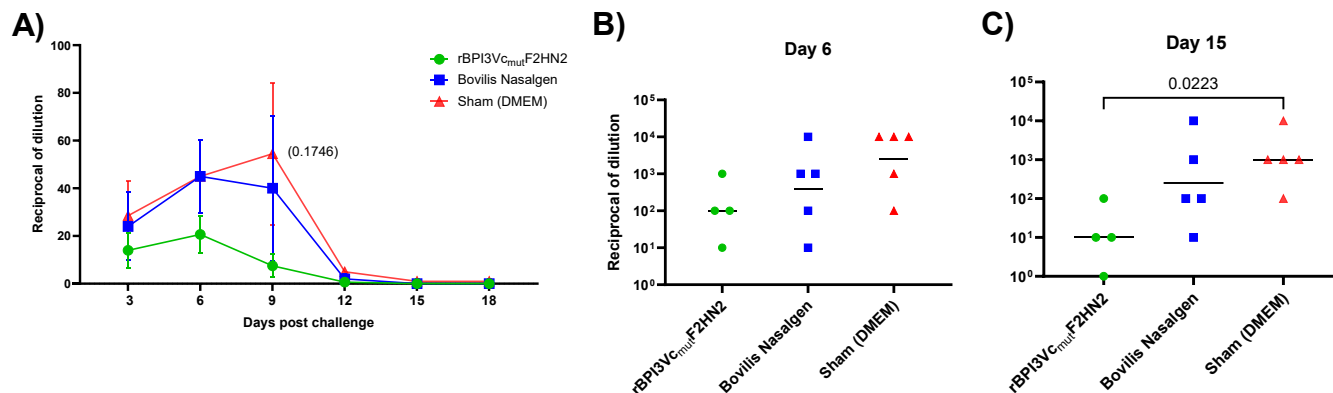


Figure 2.11. Post-challenge Viremia.

A) Nasal shedding was determined in nasal swabs collected from treatment and control calves from 3-18 days post-challenge. Mean group dilutions are plotted. Viremia was determined in blood samples collected from treatment and control calves on day 6 (B) and day 15 (C) post-challenge. Mean group dilutions are represented by the plotted bar.



Figure 2.12. Gross lung lesions.

Pulmonary atelectasis was the most common gross lesion observed consistently in all groups, and the severity ranged from: **A)** mild in the rBPI3V_{c_{mut}}F2HN2 vaccinees (10% of right cranial lung lobe affected); **B)** moderate in the Bovilis Nasalgen treatment group (50% of right cranial lung lobe affected); and **C)** severe in the ham (DMEM) control group (80% of right cranial and middle lung lobes affected).

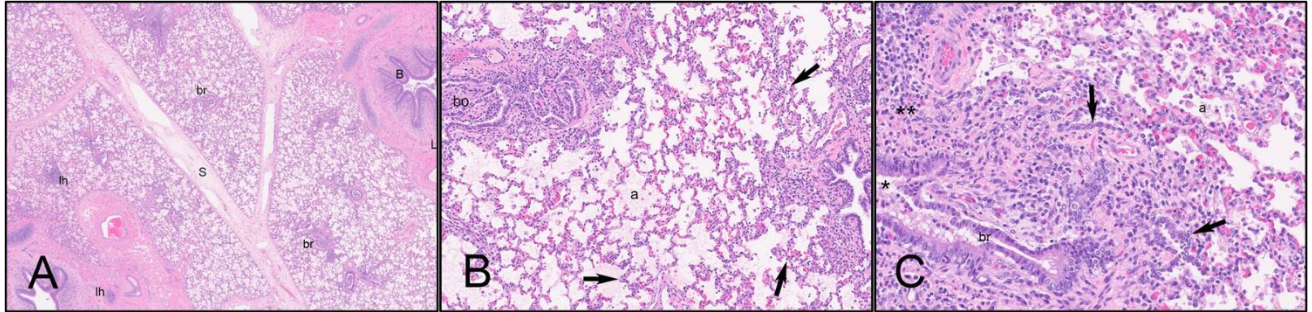


Figure 2.13. Histologic lesions.

Histologic lesions observed in calf 5669 lung [Sham negative control group] included **A)** chronic interstitial pneumonia, expansion of interlobular septa (S), peribronchial inflammation (B), lymphoid hyperplasia (lh), alveolar edema, bronchiolitis obliterans (br), and expansion of interstitial septa. 2x magnification. **B)** In more severely affected areas, there were poorly aerated alveolar spaces (a) containing edema and alveolar macrophages, bronchiolitis obliterans (bo), and chronic interstitial pneumonia (arrows). 10x magnification. **C)** In the most severely affected lobes, poorly aerated alveolar spaces (a) contained edema and alveolar macrophages, interstitial walls were expanded by inflammatory cells and occasionally are proliferative type 2 pneumocytes (arrows); bronchioles (br) contained few neutrophils (*) and were surrounded by increased fibrous connective tissue and mixed inflammatory cells (**).

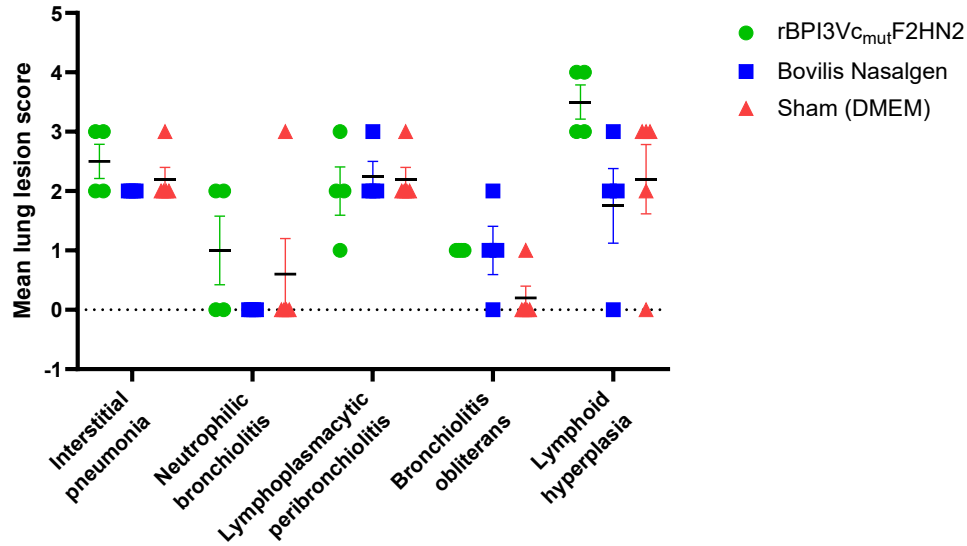


Figure 2.14. Histopathologic lung lesions.

Lung lesions were scored from sections of the cranial, middle and caudal lobes of the right and left lung, based on the severity of lesions as follows; (0= normal; 1 = lesion is present; 2 = mild; 3 = moderate; 4 = severe). Individual lung lesion score for each animal is plotted. Bars represent mean lung lesion score for each group.

Chapter 3- BPI3Vc-vectored mosaic BVDV antigens elicit broadly neutralizing antibodies in cattle

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Abstract

Bovine Viral Diarrhea Virus is a key contributor to the development of Bovine Respiratory Disease complex, which can cause respiratory disease, congenital defects, severe diarrhea, immunosuppression, abortion, or birth of persistently infected calves. Current commercial vaccines are formulated using strains from BVDV-1a and BVDV-2a genotypes, conferring protection against some, but not all, homologous genotypes and low neutralization titers against heterologous and emergent genotypes. This study aimed to develop a live vaccine capable of inducing broad protection against diverse BVDV strains. Here, a contemporary live-attenuated BPI3Vc-vectored BVDV prototype vaccine was developed. Novel mosaic BVDV E2-NS2-5¹⁻² antigens, designed based on the consensus of all BVDV-1a, -1b, -2a, -2b, and -2c protein sequences, were used to generate recombinant BPI3Vc_{mut}E2-NS2-5¹⁻² viruses. The recombinant viruses expressed all the ~5kb encoded transgenes, replicated efficiently, remained genetically stable over nine passages *in vitro*, and displayed the E2 ectodomain on the surface of infected cells. Intranasal immunization of calves with a cocktail of these recombinant viruses, designated rBPI3Vc_{mut}BVDV, elicited strong serum antibodies against BVDV genotypes 1 and 2 compared to a commercial vaccine. Notably, vaccination elicited higher IgG responses against BVDV-1b California and TGAC, with significantly higher viral neutralizing titers (California: $p=0.0031$, TGAC: $p=0.0002$) compared to the commercial vaccine. Immunized calves also elicited significantly higher VN titers against BVDV-2a strains 296NC ($p=0.0006$), 890 ($p=0.0020$), 296C ($p=0.0464$), A125 ($p=0.0018$), and 1373 ($p=0.0025$). Upon challenge with BVDV-1b California, the rBPI3Vc_{mut}BVDV vaccinees exhibited a steady weight gain, less decrease in lymphocyte counts, lower blood viral load, and fewer gross lesions. This data supports use of the live-

attenuated BPI3Vc-vector for development of contemporary broadly protective BVDV vaccines that can easily be upgraded for improved disease management and cattle productivity.

3.1 Introduction

Bovine viral diarrhea (BVD) is an infectious disease caused by the Bovine viral diarrhea virus, which affects cattle and other ruminants globally (Richter et al., 2017). The BVDV is a key contributor to the development of Bovine Respiratory Disease complex (BRDC). Infection with the virus can lead to severe diarrhea, respiratory disease, immunosuppression, abortion, congenital defects, and birth of persistently infected (PI) calves, which serve as a major reservoir and shedder of the virus within a herd (Campbell, 2004; Loneragan, 2005). Immunosuppressed calves are particularly vulnerable to secondary bacterial and fungal infections, which may result in pneumonia or enteritis (Baule et al., 2001; Chase, Elmowalid, & Yousif, 2004). These secondary infections contribute to high rates of morbidity and mortality in affected animals, resulting in multi-billion dollar losses by the U.S. livestock industry due to BRDC (R. A. Smith, 2009).

The pathogen is an enveloped single-stranded RNA virus classified under the genus *Pestivirus* in the family *Flaviviridae* (D. B. Smith et al., 2017). Its genome is approximately 12.5 kb, containing a single open reading frame that encodes for a 450 kDa precursor polyprotein. This polyprotein undergoes proteolytic processing to yield both structural and non-structural proteins. The structural proteins consist of N^{pro}, capsid protein, and glycoproteins E^{ms}, E1, and E2, whereas non-structural proteins include the NS2-3, NS4A-B, and NS5A-B (Rumenapf, Unger, Strauss, & Thiel, 1993). The virus is classified into three genotypes, recently classified as different species, based on antigenic differences: BVDV Type 1, which consists of 22 sub-genotypes (BVDV-1a to 1v); BVDV Type 2, which consists of 4 sub-genotypes (BVDV-2a to 2d); and BVDV Type 3, which consists of 4 sub-genotypes (BVDV 3a to 3d) (L. Liu, Xia, Wahlberg, Belák, & Baule, 2009; Postler et al., 2023;

Yeşilbağ, Alpay, & Becher, 2017; Zhu et al., 2022). Additionally, the virus is categorized into cytopathic or non-cytopathic biotypes, based on the presence or absence of cytopathic effects, respectively, in infected cell cultures (Julia F Ridpath, 2005). *In vivo*, non-cytopathic biotypes are linked to the development of persistent infection while cytopathic biotypes are associated with the occurrence of mucosal disease (Moennig & Becher, 2018; Julia F Ridpath, 2005).

The mainstream BVDV control strategy includes biosecurity practices, diagnosis and elimination of persistently infected animals, and vaccination (K. V. Brock, 2004). Vaccination programs primarily utilize Modified Live Virus vaccines (MLVs) or Killed Virus vaccines (KVs), with several commercial vaccines available that have been commercially available for decades. The most recognized genotypes in North America were considered to be BVDV-1a, -1b, and -2a (Robert W Fulton et al., 2006; Julia F Ridpath, Fulton, Kirkland, & Neill, 2010). Therefore, almost all MLVs or KVs are currently formulated using a combination of one representative strain from BVDV-1a and one from BVDV-2a (Robert W Fulton, Cook, Payton, Burge, & Step, 2020; Melkamsew, Tessema, & Paeshuyse, 2025). Interestingly, BVDV-1b is now the most predominant species in the United States (Robert W Fulton et al., 2006; Julia F Ridpath et al., 2010; Julia F. Ridpath et al., 2011; Stronga, 2013). Additionally, BVDV genotypes 2b and 2c were recently identified, with genetic and antigenic differences from BVDV-2a (Neill et al., 2019). Notably, studies indicate that sera from cattle immunized with various BVDV-2a MLV vaccines varied in ability to neutralize BVDV-2b and 2c strains. Specifically, this study reported that the vaccine strains elicit protective neutralization titers against some, but not all, BVDV-1a, -1b, and -2a strains and significantly low neutralizing titers against BVDV-2c strains (Robert W Fulton et al., 2020). Similar outcomes have been observed in other studies, where genotype-specific MLV vaccines were ineffective at conferring cross-protection against diverse BVDV strains, in Brazilian (Baccili et al., 2019; Botton, Da-Silva, Brum, Weiblen, & Flores, 1998)

and European livestock (Alpay & Yeşilbağ, 2019; Grange, Mindeguia, Gisbert, & Meyer, 2023). This strongly suggests that there are significant antigenic differences between some commercial vaccines and circulating field strains. Current MLV vaccines do not provide broad epitope coverage of the diverse circulating BVDV strains. Additionally, each BVDV strain has shared and unique neutralizing epitopes, and thus, a vaccine that can capture this diverse epitope repertoire is expected to confer broad protection.

Colostrum-derived BVDV-specific neutralizing antibodies provide the initial control against BVDV infection in neonates and yearlings (Brar, Johnson, Muscoplat, Shope Jr, & Meiske, 1978). However, as the neutralizing antibody titers decay, calves become vulnerable to infection at different time points, and in the presence of PI calves in the herd, there is always a high risk of infection (Robert W. Fulton et al., 2004). Vaccination activates both adaptive antibody and T cell-mediated immunity, whereby neutralizing antibodies block infections caused by wild-type BVDV, while CD4⁺ T cells help induce and maintain CD8⁺ cytotoxic T cells necessary for the clearance of infected cells, conferring long-term protection (Beer, Wolf, Pichler, Wolfmeyer, & Kaaden, 1997; Howard, Clarke, Sopp, & Brownlie, 1992; Julia F Ridpath, 2013). Current MLVs and KVs are administered parenterally (Falkenberg, Dassanayake, Crawford, Sarlo Davila, & Boggiatto, 2023; Grange et al., 2023; Masic et al., 2021; Melkamsew et al., 2025; Taberner et al., 2024), with the MLVs having the advantage of replicating *in vivo* and thereby mimicking wild-type virus infection. However, in calves less than six months old, parenterally administered vaccines are highly susceptible to maternal antibody neutralization and complement-mediated clearance that limits their efficacy (Ellis, 2001; R. W. Fulton, J. F. Ridpath, S. Ore, A. W. Confer, J. T. Saliki, L. J. Burge, and M. E. Payton., 2005.; Zimmerman, Boots, Valli, & Chase, 2006). Consequently, immunization with BVDV-1 and -2 vaccines is usually delayed for about six months to allow maternal antibodies to wane. Unfortunately, in the presence of PI calves in the herd,

there is always a high risk of infection as antibodies decay below protective titers earlier than six months (Brar et al., 1978; Robert W. Fulton et al., 2004; Howard et al., 1992). Additionally, some calves entering feedlots are PI, and the lack of effective chute-side testing to identify them poses a great risk to unprotected cattle (Robert W Fulton et al., 2006). Therefore, improved vaccines are needed to ensure a more consistent control of BVDV infection in calves.

Rationally designed live-vectored BVDV subunit vaccines that can be delivered intranasally offer an attractive alternative in that the live vector can replicate in the respiratory tract, the site for BVDV infection, priming BVDV-specific immune responses in the presence of neutralizing maternal antibodies. Protective BVDV antigens have been identified. The E2 and NS2-3 antigens are immunodominant, and neutralizing antibodies as well as T cell responses against these antigens can confer protection against wild-type virus (Deregt et al., 2005; Lokhandwala et al., 2017; Sangewar et al., 2021; van Oirschot, 1999). Additionally, MHC *DR*-restricted T cell epitopes have been identified from conserved regions of E2 and NS2-3 (Beer et al., 1997; Collen, 2000.; Deregt et al., 2005; Hegde, 1997; Howard et al., 1992). In addition to E2 and NS2-3, the NS4-5 antigens contain CD4⁺ T-cell epitopes that induce strong IFN- γ secretion (Collen, 2000.; Riitho et al., 2017), and novel potent IFN- γ -inducing CD8⁺ T cell epitopes that are highly conserved among diverse BVDV strains have been identified (Sangewar et al., 2021).

Certain regions of the E2, NS2-3, and NS4-5 antigens are conserved among BVDV strains (Deng, 1992). However, these antigens, particularly E2, also contain variable regions within antigen domains that are crucial for eliciting protective immunity, thus contributing to the variable cross-reactivity observed among BVDV strains which, in part, explains why current vaccines are poor at conferring cross-protection against diverse BVDV strains (Botton et al., 1998). A vaccine containing all defined protective B and T cell epitopes from diverse BVDV-1 and -2 strains is yet to be developed

and tested in calves. We hypothesized that a rationally designed prototype vaccine containing E2, NS2-3, and NS4-5 mosaic antigens, which incorporates protective epitopes conserved among BVDV-1a, 1b, and BVDV-2 (a, b, c) genotypes as well as strain-specific protective epitopes, would confer broad protection against BVDV-1 and -2 strains.

In this study, a contemporary live attenuated Bovine parainfluenza 3 virus vector, designated BPI3V_{Cmut}, encoding novel mosaic BVDV E2-NS2-5¹⁻² antigens, was developed based on the consensus of more than 200 sequenced BVDV-1 and -2 genomes in the United States as of 2019. The E2 structural glycoproteins were designed for multicistronic expression and surface display on infected cells for optimal B cell recognition. A cocktail of the recombinant BPI3V_{Cmut}-vectored BVDV antigens, designated rBPI3V_{Cmut}BVDV, was evaluated for its multicistronic antigen expression, surface display of the E2 structural antigens, immunogenicity, and protective efficacy in five-month-old Holstein calves following challenge.

3.2 Materials and Methods

3.2.1 Cells and viruses

Baby Hamster Kidney cells constitutively expressing T7 RNA polymerase (BSR T7/5 cells) and Madin–Darby bovine kidney (MDBK) cells (Millipore Sigma, MDBK NBL-1) were maintained as previously described (Chapter 2). Wild-type BVDV-1b (strains California (CA0401186a) and TGAC) and BVDV-2a (strains A125 and 1373) viruses were obtained from RTI (Dr. Chris Chase). BVDV viruses were propagated in MDBK cells at 37°C.

3.2.2 Monoclonal and Polyclonal antibodies

Mouse anti-Flag tag monoclonal antibody (mAb) and mouse anti-His tag mAb were sourced from Millipore Sigma (F1804) and Invitrogen (MA1-21315), respectively. The mouse mAb against BVDV-1 and -2 E2 glycoprotein was sourced from VMRD (Cat # 348, Pullman WA). Polyclonal antibody (pAb) 2539 against BVDV-1 and -2 was purified by protein-G affinity chromatography from sera of a steer hyperimmunized with Gamma irradiated BVDV-1b TGAC (Sangewar et al., 2021). The AR22.7 mouse mAb against *Theileria parva* modified surface protein was obtained from Washington State University (Tebaldi et al., 2017). The mAbs were used at 4 µg/ml, whereas the purified pAbs (IgG) were used at 6 µg/ml for Immunofluorescence and Immunocytometric analyses, respectively. For Western blots, the mAbs were used at 1.5 µg/ml.

3.2.3 Design and generation of plasmid constructs

The pFLC-BPI3Vc_{mut} plasmid construct containing the BPI3Vc_{mut} backbone was previously described (Chapter 2). Five novel mosaic BVDV antigen expression cassettes, designated E2-NS2-3^{1,2,3} (E2-NS2-3¹⁻³), containing consensus mosaic E2-NS2-3 determinants from sequenced BVDV-1a, -1b, and BVDV-2 genotypes, respectively, and NS4-5^{1,2} (NS4-5¹⁻²), containing consensus determinants from sequenced BVDV-1 and BVDV-2 genotypes, respectively, were designed. All five expression cassettes encoding E2-NS2-3¹⁻³ and NS4-5¹⁻², collectively designated as E2-NS2-5¹⁻², were codon-optimized, synthesized, and cloned into the pFLC-BPI3Vc_{mut} plasmid. The resultant constructs were used to rescue recombinant viruses that were tested for protein expression as previously described (Chapter 2).

Briefly, all available E2-NS2-5¹⁻² protein sequences from North American BVDV strains as of 2019 were retrieved from NCBI, including 41 sequences encoding E2-NS2-3¹, 51 sequences

encoding E2-NS2-3², 112 sequences encoding E2-NS2-3³, 77 sequences encoding NS4-5¹, and 101 sequences encoding NS4-5². Multiple sequence alignment performed using MEGA 7 generated consensus sequences based on a > 50% identity threshold. The dominant amino acid was selected when identity was less than 50%. The final consensus sequences for the mosaic E2-NS2-3² and NS4-5² polypeptides were used to generate maximum likelihood phylogenetic trees. The resulting consensus FASTA files were then analyzed to evaluate the conservation levels of mosaic E2-NS2-5¹⁻² sequences compared to their respective protein sequences.

The E2¹⁻³ and NS2-3¹⁻³ protein sequences were designed as multicistronic expression cassettes, separated by a 2A autocleavable motif (Ryan, King, & Thomas, 1991), while the NS4-5¹⁻² protein sequences were designed as monocistronic expression cassettes. In addition, genes encoding an irrelevant antigen, *Theileria parva* modified surface protein (TMSP) (Tebaldi et al., 2017), were also designed as a monocistronic expression cassette. To enable antigen display on the surface of infected cells, sequences encoding the transmembrane and cytoplasmic domains of BPI3Vc Fusion protein were added, in-frame, downstream of the mosaic E¹⁻³ and TMSP protein sequences. A Flag tag sequence (DYKDDDDK) was added in-frame, at the N-terminal, and a His tag sequence (HHHHHH) at the C-terminal, of each of the mosaic E2-NS2-5¹⁻⁵ and the TMSP protein sequences. The resulting polypeptide sequences were used to generate multicistronic and monocistronic expression cassettes, which were codon optimized for *Bos taurus* expression and cloned into pCDNA 3.1 (+) mammalian expression vector to generate constructs designated as pCDNA-E2-NS2-5¹⁻² (GenScript). Protein expression by these constructs was evaluated by Immunocytometric analysis using an anti-Flag mAb-AP (Alkaline phosphatase-conjugated) (Millipore Sigma, F3165), an anti-His mAb, mAb 348 or pAb 2539, followed by an anti-mouse or

anti-bovine IgG-AP-labeled secondary antibodies, respectively (Jackson ImmunoResearch, 115-055-003, 101-055-003).

Genes from the pCDNA-E2-NS2-5¹⁻² subclones with the best protein expression along with a previously validated pCDNA-TMSP construct (GenScript), were used as templates for PCR amplification and subcloning of transgenes into the pFLC-BPI3V_{cmut} plasmid to generate recombinant plasmid constructs, designated pFLC-BPI3V_{cmut}E2-NS2-5¹⁻² and pFLC-BPI3V_{cmut}TMSP, which were used for virus assembly. Briefly, genes encoding the mosaic E2-NS2-3¹⁻³ were PCR amplified using the following primers; Forward-1: 5' GTAAGAAAACTTAGGATTAACGGAGCCGCCACCATGGGATCCACGGCCGCCGCCGC CATGGCCATCGCGTATGATCACCATAGTTG 3'; Forward-2: 5' GATTCTCGTATCGTAT CTGGCGCGCCAAGTAAGAAAACTTAGGATTAACGGAGCCGC 3'; and Reverse: 5' CGTGATAGTGGCGCGCCGCTAGCTATAGCGGCCGCATCATTACTAGTGGTGTATGGTGA TG 3'. Mosaic NS4-5¹ gene was PCR amplified using the following primers: Forward-1: 5' GAAAACTTAGGATTAACGGAGCCGCCACCATGGGATCCACGCGTGCCGCCGCCGCC ATGGCCATGGACTACAAGGACGATGACGATAAG 3'; Forward-2: 5' GATTCTCGTA TCGTATCTGGCGCGCCAAGTAAGAAAACTTAGGATTAACGGAGCCGC 3'; and Reverse: 5' CGTGATAGTGGCGCGCCGCTAGCTATAGCGGCCGCATCATTACTAGTG GTGATGGTGTATG 3'. Mosaic NS4-5² gene was PCR amplified using the following primers; Forward-1: 5' GAAAACTTAGGATTAACGGAGCCGCCACCATGGGATCCACGC GTGCCGCCGCCGCCATGGCCATGGACTACAAGGACGATGACGATAAG 3'; Forward-2: 5' GATTCTCGTATCGTATCTGGCGCGCCAAGTAAGAAAACTTAGGATTAACGGAGCCGC 3'; and Reverse: 5'CGTACTGGCGCGCCGCTAGCTATAGCGGCCGCATTATCAT TACTAGTGGTGTATGGTGTATG 3'. TMSP gene was PCR amplified using the following primers;

Forward-1: 5' ACTGTGATAATAGTACGCGTGCCGCCGCCGCCATGGCCATGGCCATG ACCACCATAGTTG 3'; Forward-2: 5' TAAGAAAACTTAGGATTAACGGAGCCGC CACCATGGGATCCACGCGTGCCGCCGCCGC 3'; Forward-3: 5' GATTCTCGTATCGTAT CTGGCGCGCCAAGTAAGAAAACTTAGGATTAACGGAGCCGC 3'; TMSP Reverse-1: 5'AGCTGTAGTGATAATAGTGCGGCCGCATTATCATTACTAGTGGTGATGGTGATGATG 3'; and Reverse-2: 5' CGTACTGGCGCGCCGCTAGCTATAGCGGCCGCATTATCAT TACTAGTGGTGATGGTGATG 3'. The primers introduced an AscI restriction site along with essential elements for cloning transgenes between the N and P genes of the backbone, while maintaining the 'rule of six' (Kolakofsky et al., 1998), to ensure efficient transgene expression and virus replication, as previously described (Chapter 2). The resulting PCR products were cloned into the pFLC-BPI3V_{Cmut} backbone, as previously described (Chapter 2), and positive constructs were identified by PCR colony screening using a vector-specific Forward primer (5' AGGGCAG CCTGAATCCAGAGGAGATCAGGATCA 3') and gene-specific reverse primers; E2-NS2-3^{1,2} Reverse primer: (5' TATGGACGATGACGATAAGTCCCTCGAGGCCAGTCCCACCACCTG CTTCAG 3'); E2-NS2-3³ Reverse primer: (5' TATGGACGATGACGATAAGTCCCTCGAGG CCAGGCCGGTCACCTGCTTCAG 3'); NS4-5¹ Reverse primer: 5' CGTGATAGTGGCGCGC CGCTAGCTATAGCGGCCGCATCATTACTAGTGGTGATGGTGATG 3'; NS4-5² Reverse primer: 5' CGTACTGGCGCGCCGCTAGCTATAGCGGCCGCATTATCATTACTAGTGGTG ATGGTGATG 3' or TMSP Reverse primer: 5'AGCTGTAGTGATAATAGTGCGGCCGCATT ATCATTACTAGTGGTGATGGTGATGATG 3'. AscI restriction digest of positive pFLC-BPI3V_{Cmut}E2-NS2-5¹⁻² and pFLC-BPI3V_{Cmut}TMSP plasmid minipreps was used to confirm presence of ~5 kb or ~2.6 kb transgenes, respectively, and sequencing was used to validate gene integrity.

3.2.4 Generation and validation of recombinant viruses

Recombinant viruses were assembled by co-transfecting BSRT T7/5 cells with 3 µg of the antigenome plasmid constructs (pFLC-BPI3V_{cmut}E2-NS2-5¹⁻², pFLC-BPI3V_{cmut}TMSP) along with 1.6 µg, 1.6 µg, and 0.8 µg of pCDNA-N, pCDNA-P, and pCDNA-L helper plasmids, respectively. Viruses were rescued by co-culture of the transfected BSRT T7/5 cells as previously described (Chapter 2), to generate recombinant viruses designated rBPI3V_{cmut}E2-NS2-5¹⁻² and rBPI3V_{cmut}TMSP, respectively. Cell pellets and supernatants were processed to generate seed viral stocks, which were snap-frozen at -80°C. A ten-fold diluted stock virus was used to validate protein expression by Immunocytometric analysis or Immunofluorescence assay (IFA) as previously described (Chapter 2). Protein expression by the rBPI3V_{cmut}E2-NS2-5¹⁻² or the rBPI3V_{cmut}TMSP viruses was validated using the anti-Flag mAb-AP, anti-His mAb, the mAb 348 against BVDV-1 and -2 E2 glycoprotein, the bovine pAb 2539 against BVDV, or the mAb AR22.7 against TMSP. Secondary antibodies used for IFA, including a goat anti-mouse IgG-FITC (Jackson ImmunoResearch) and a goat anti-bovine IgG-FITC (Jackson ImmunoResearch), were diluted at 1:1000. Uninfected wells were included as negative controls. Images of the cells expressing the target proteins were captured using an AmScope MU1803-HS digital camera, and the best-expressing clone for each construct was selected for virus expansion in MDBK cells followed by virus purification over a 30-70% sucrose gradient, as previously described (Chapter 2).

Expression of the ~ 53 kD E2¹⁻³, ~ 135 kD NS2-3¹⁻³ antigens by the multicistronic cassettes, or the ~ 185 kD NS4-5¹⁻² and ~87 kD TMSP antigens by the monocistronic cassettes, was confirmed by Western blot analysis, as previously described (Chapter 2), but by using 1.5 µg/ml mAb 348 followed by a goat anti-mouse IgG-HRP diluted at 1:1000 (Jackson ImmunoResearch), 1.5 µg/ml of anti-Flag mAb-HRP (Sigma Aldrich), or 1.5 µg/ml of mouse anti-His mAb

(Invitrogen) followed by a goat anti-mouse IgG-HRP diluted at 1:1000 (Jackson ImmunoResearch).

3.2.5 Flow cytometric analysis

The E2¹⁻³ and TMSP polypeptides, designed with the transmembrane and cytoplasmic domains of BPI3Vc Fusion protein to anchor the E2 and TMSP ectodomains on the surface of infected MDBK cells, were analyzed by Flow cytometry as previously described (Chapter 2). Briefly, 80% confluent 6-well MDBK cells were infected with the rBPI3Vc_{mut}E2-NS2-3¹⁻³, the rBPI3Vc_{mut}TMSP, or an irrelevant rBPI3Vc_{mut}COV19 Spike virus at an MOI of 1. The infected cells were incubated at 37°C for 2 days, then harvested and processed for analysis, as previously described (Chapter 2). Surface staining was performed using mAb 348 against E2¹⁻³ (1:250 dilution) or mAb AR22.7 against TMSP (1:250 dilution), followed by a goat anti-mouse IgG-FITC (1:250 dilution). Data was acquired and analyzed as previously described (Chapter 2).

3.2.6 Viral growth curves

Multicycle replication growth curves of representative recombinant viruses expressing BVDV-1 and -2 antigens, including negative control viruses, were performed as previously described (Chapter 2). Briefly, 80% confluent 6-well MDBK cells were infected at an MOI of 1 in duplicates with the viruses: i) rBPI3Vc_{mut}E2-NS2-3¹, ii) rBPI3Vc_{mut}NS4-5², iii) rBPI3Vc_{mut}TMSP, or iv) wild-type BPI3Vc TVMDL16 virus. The infected cells were incubated at 37°C for four days. Cultures were gently mixed every 24 hours, and a 0.5 ml sample was aliquoted and replenished with an equal volume of fresh media. The virus aliquots were snap-frozen at -80°C, and virus titers from each time point were determined by TCID₅₀ calculation as previously described (Chapter 2).

3.2.7 Viral RNA extraction and sequencing

MDBK cells grown in 6-well plates were infected at 80% confluence with representative Passage 1 (P1) viruses expressing BVDV-1 and -2 antigens, including rBPI3V_{cmut}E2-NS2-3^{1,2} and rBPI3V_{cmut}NS4-5², at an MOI of 1, and incubated at 37°C for four days. The resulting P2 virus was diluted ten-fold and infected onto a fresh MDBK monolayer. After nine passages, cell pellets from the P2 and P9 viruses were resuspended in 1ml Trizol (Thermo Fisher Scientific, 15596026), RNA extraction and sequenced using the Illumina platform as previously described (Chapter 2). The percentage of sequence identity of the P9 progeny viruses was compared to P2 progenies and the original plasmid sequences.

3.2.8 Immunization

Fifteen five-month-old male Holstein calves (obtained from J.R. Livestock Inc, Iowa), seronegative for BVDV-1 and -2 as confirmed by sera neutralization tests (performed by Iowa State University Veterinary Diagnostic Laboratory), were included in this study. The calves were randomly allocated to three groups (n=5) (Table 3.2). Seven days after acclimatization, each calf in group 1 was immunized intranasally with the rBPI3V_{cmut}E2-NS2-5¹⁻² virus constructs, designated rBPI3V_{cmut}BVDV (4.4×10^9 TCID₅₀), followed by a first and second booster dose of 4.4×10^9 TCID₅₀ and 8.4×10^{10} TCID₅₀, administered on week 3 and 6 post-priming, respectively. All intranasal immunizations were conducted using an intranasal mucosal atomization device (Teleflex MAD 300). Each calf in group 2 received a subcutaneous injection of the recommended 2ml commercial Bovi-Shield Gold 5 vaccine (Zoetis) for the prime and booster doses. The commercial vaccine is a multivalent Modified Live Virus (MLV) vaccine against Infectious bovine rhinotracheitis, Bovine viral diarrhea virus 1 and 2, Parainfluenza 3 virus, and Bovine respiratory

syncytial virus. Calves in group 3 served as negative controls. They were immunized intranasally with the irrelevant rBPI3V_{Cmut}TMSP virus at 1.4×10^9 TCID₅₀ for the priming dose, followed by a first and second booster dose of 2.9×10^9 TCID₅₀ and 1.2×10^{10} TCID₅₀, respectively. All priming and booster viruses were back-titrated after administration. The three groups of calves were housed separately during the immunization phase. Their rectal temperature and body weights were recorded weekly.

3.2.9 Evaluation of antibody responses

Coagulated blood was collected at different time points (Fig. 3.6), and sera antibodies were assayed for recognition of BVDV wild-type viruses by IFA, then the end-point dilution titers were determined. Briefly, the coagulated blood was centrifuged to obtain sera. MDBK cells grown in 6-well plates were infected at 80% confluency in duplicates with the BVDV-1b strains (California, TGAC) or BVDV-2a strains (A125 and 1373) at an MOI of 1. Uninfected wells were included as negative controls. After incubation for three days at 37°C, the plates were fixed and probed with sera collected one week after the second boost, diluted at 1:100, followed by a goat anti-bovine IgG-FITC (1:1,000 dilution, Jackson ImmunoResearch). Plates were evaluated using an Olympus CKX53 fluorescent microscope, and images were captured using an AmScope MU1803-HS digital camera. To determine the endpoint dilution titer, MDBK cells were grown in 96-well plates to 80% confluency and infected with the BVDV-1b strains (California, TGAC) or BVDV-2a strains (A125 and 1373) as described above for three days at 37°C. Plates were fixed and probed with sera from all the calves collected one week after the second boost as described above. Sera were serially diluted two-fold from 1:250 to 1:4000 and used to probe infected cells in duplicates. Antibody titers are expressed as the reciprocal of the highest serum dilution where staining was observed.

3.2.10 Virus neutralization

Neutralization of wild-type BVDV-1a (Singer, NADL), BVDV-1b (California (CA0401186a), TGAC), or BVDV-2a (1373, 296NC, 890, 296C, A125) viruses was evaluated using sera from blood collected one week after the second boost as previously described (Chapter 2). The presence or absence of virus in the cytopathic BVDV-1 (Singer, NADL, and TGAC) and BVDV-2 (A125, and 296C) was confirmed by observing CPE. Presence of virus in the non-cytopathic BVDV-1 (BJ, and CA0401186a) and BVDV-2 (890,1373, and 296NC) was confirmed by immunoperoxidase assay (R. Fulton et al., 1997). Virus neutralization titers were expressed as the reciprocal of the highest dilution in which more than 50% CPE or staining for the virus was detected.

3.2.11 Animal challenge

Sixty days after priming, all the calves were challenged by intranasal instillation of 3.4×10^5 TCID₅₀ of BVDV Type 1b California, CA0401186a virus (Sangewar et al., 2020), with the challenge virus back-titrated after administration. BVDV-1b is the most prevalent strain in North America and is recommended for conducting BVDV vaccine efficacy studies (Julia F Ridpath, Neill, & Peterhans, 2007). The calves were monitored daily for 18 days for clinical signs, including changes in breathing, liveliness, ocular and nasal discharges, coughing, changes in appetite, diarrhea, and any indications of depression. Rectal temperatures were recorded daily, and body weights were measured before challenge, six- and twelve-days after challenge. Uncoagulated blood was collected every three days. After 18 days, all animals were humanely euthanized for necropsy following approved protocols at Kansas State Veterinary Diagnostic Laboratory.

3.2.12 Hematology and viremia

On days 0, 3, 6, 9, 12, 15, and 18 post-challenge, uncoagulated blood was drawn into K2 EDTA-coated tubes to evaluate white blood cell counts, lymphocyte counts, and viremia. As previously described, automated hematology analysis was performed at Kansas State University Veterinary Diagnostic Laboratory (Chapter 2). Hematologic parameters, including White Blood Cell (WBC) and Lymphocyte (LYMPH) counts, were verified by two staff personnel and recorded. To establish a common baseline, the mean WBC or Lymphocyte count for each group on DPC 3, 6, 9, 12, 15, and 18 were subtracted from their respective initial values on DPC 0, and the resulting differences were plotted as mean count ratios relative to baseline.

Titers of BVDV-1b California wild-type virus in blood after challenge were determined by titrating samples in MDBK cells followed by IFA, as described in previous studies (K. Brock, Grooms, Ridpath, & Bolin, 1998; Lokhandwala et al., 2017; Sangewar et al., 2020). Briefly, blood samples underwent three freeze-thaw cycles and were centrifuged, and the resulting lysates were diluted ten-fold in DMEM. 96-well MDBK cell monolayers were then infected with 50 µl of each serially diluted lysate, with nine replicate wells per sample. After incubation at 37°C for five days, the plates were fixed in formalin and stained using the previously mentioned mAb 348 and a goat-anti mouse IgG-FITC (Jackson ImmunoResearch). The BVDV titer was determined as the highest dilution at which positive staining of the MDBK cells was observed.

3.2.13 Necropsy and Pathology

Three weeks after challenge, the study was terminated and all calves were sedated using xylazine and euthanized by penetrative captive bolt. Complete necropsy examination, analysis of gross lung lesions, and histopathologic assessment of the cranial, middle, and caudal lobes of the right lung

and the cranial and caudal lobes of the left lung were performed at Kansas State University Veterinary Diagnostic Laboratory, as previously described (Chapter 2). Morphologic diagnosis of histologic lesions was graded as mild, moderate, or severe and assigned scores reflecting the presence and severity of each lesion as (0= normal; 1 = lesion is present; 2 = mild; 3 = moderate; 4 = severe).

3.2.14 Statistical analysis

GraphPad Prism10 was used to analyze data. The significance of differences in mean antibody titer between the three treatment groups during the different sampling time points was analyzed by Two-way repeated measures ANOVA followed by Tukey correction for multiple mean comparisons. For the blood viremia data analysis, the significance of differences between the treatment and control groups was analyzed by One-Way ANOVA followed by Dunnett's T3 correction for multiple comparisons. Statistical differences with significance levels of $p < 0.05$ was considered significant.

3.3 Results

3.3.1 Recombinant viruses expressed mosaic BVDV antigens

The live attenuated BPI3V genotype-C vector (BPI3V_{Cmut}) was described as a replicon with unique cloning sites for cloning transgenes of interest (Chapter 2). Here, novel mosaic BVDV E2-NS2-5¹⁻² polypeptide sequences were generated based on the consensus of over 200 sequenced BVDV-1 and -2 genomes. Specifically, mosaic E2-NS2-3¹⁻³ sequences were derived from the consensus of 44, 51, and 112 genomes for BVDV-1a, BVDV-1b, and BVDV-2, respectively, whereas mosaic NS4-5¹⁻² sequences were generated from the consensus of 77 and 101 genomes for BVDV-1 and

BVDV-2, respectively (Fig. 3.2A and B). The mosaic sequences exhibited varying conservation levels with their corresponding protein sequences. Specifically, the mosaic E2² and NS2-3² sequence were 75.6% and 93.2% identical to the 51 E2 and NS2-3 BVDV-1b protein sequences, respectively (Fig. 3.2C). The mosaic NS4-5² sequence displayed a 70.7% identity to the 101 NS4-5 BVDV-2 protein sequences (Fig. 3.2D). Additionally, the mosaic polypeptides for E2¹ and NS2-3¹, E2³ and NS2-3³, and NS4-5² showed sequence identities of 36.3% and 77.9%, 57.3% and 75.6%, and 72.8% with the corresponding BVDV E2-NS2-3¹, E2-NS2-3³, and NS4-5² proteins, respectively. This indicates that the E2 glycoprotein was the most heterogeneous, suggesting that the mosaic sequence contains shared and unique neutralizing epitopes.

Recombinant pCDNA constructs generated using codon-optimized synthetic genes encoding the mosaic BVDV antigens, designated pCDNA-E2-NS2-5¹⁻², expressed the antigens that were recognized by anti-Flag mAb (Fig. 3.3A), anti-His mAb, mAb 348, and pAb 2539 (data not shown). The genes encoding the multicistronic E2-NS2-3¹⁻³ cassettes, as well as the monocistronic NS4-5¹⁻² and TMSP cassettes, were used to generate recombinant viruses, designated as rBPI3V_{cmut}E2-NS2-5¹⁻² and rBPI3V_{cmut}TMSP, as described in chapter 2. Expression of the encoded antigens was confirmed by Immunocytometric analysis, IFA (Fig. 3.3A) and Western blots (Fig. 3.3B), using anti-Flag mAb to detect E2¹⁻³, anti-His mAb to detect NS2-3¹⁻³, mAb 348 to authenticate BVDV-1 and -2 E2¹⁻³ antigen, anti-Flag mAb to detect the Flag-tag located at the N-terminal of NS4-5¹⁻² and TMSP, anti-His mAb to detect the His tag located at the C-terminal of NS4-5¹⁻² and TMSP, pAb 2539 BVDV to authenticate NS4-5¹⁻², and mAb AR22.7 against TMSP. In addition, flow cytometric analysis showed that the rescued rBPI3V_{cmut}E2-NS2-3¹⁻³ and rBPI3V_{cmut}TMSP viruses, but not the cognate control rBPI3V_{cmut}COV19 Spike virus,

displayed the E2¹⁻³ and TMSP antigens, respectively, on the surface of infected MDBK cells (Fig. 3.4).

3.3.2 rBPI3V_{Cmut} vectored viruses exhibit genetic stability

Replication of the rescued recombinant viruses was not inhibited by the presence of transgenes. Specifically, multicycle replication growth curves demonstrated that the rBPI3V_{Cmut}E2-NS2-3¹, the rBPI3V_{Cmut}E2-NS4-5², and the rBPI3V_{Cmut}TMSP viruses replicated in a similar manner, with titers of 8.35 TCID₅₀/ml, 8.16 TCID₅₀/ml, and 7.8 TCID₅₀/ml recorded on day four, respectively (Fig. 3.5). These titers were comparable to the 8.45 TCID₅₀/ml titers recorded for the wild-type BPI3Vc TVMDL16 virus on the same day, suggesting that the presence of the ~2.6 kb TMSP or ~5 kb E2-NS2-3^{1a} and NS4-5² transgenes did not affect overall virus replication (Fig. 3.5).

The stability of transgene expression *in vitro* was analyzed by passaging the rBPI3V_{Cmut}E2-NS2-3¹, the rBPI3V_{Cmut}E2-NS2-3², and the rBPI3V_{Cmut}NS4-5² viruses in MDBK cells several times and sequencing the antigenome of resulting viral progenies. After nine passages, sequences encoding the N, P, and M genes of the BPI3V_{Cmut} backbone remained identical to those of the original sequence and the initial P2 progenies in all three viruses (Table 3.1). For the rBPI3V_{Cmut}E2-NS2-3¹ virus, nine rounds of passaging resulted in no change to the F and HN genes, a L120 P mutation in the E2¹ gene, and a V1030Q mutation in the NS2-3¹ gene. Notably, T-to-C point substitutions were observed downstream of the Flag tag of the E2 gene in only the P2 progeny, but not the P9 progeny, which was identical to the original sequence, suggesting that the expanded virus was non-clonal. For the rBPI3V_{Cmut}E2-NS2-3² virus, nine rounds of passaging resulted in no change to the F, HN, and E2-NS2-3² genes. A N556D mutation was observed in the HN gene of the P2 virus, but not the P9 virus, which was identical to the original sequence.

Additionally, mutations in the E2² gene (Y3H, Y8H, Y10H) and a P689S mutation in the NS2-3^{1b} gene of the P2 virus were carried forward to the P9 progeny. For the rBPI3V_{cmut}NS4-5² virus, nine rounds of passaging resulted in a G324S mutation in the F gene of the P9 virus and some mutations (I25S, I28S, M27T, L39P, V1261A) in the NS4-5² gene. Notably, a G218R mutation in the HN gene of the P2 virus was passed on to the P9 progeny. The L gene remained unchanged across all viruses after the nine passages, except for a consistent T-to-A point substitution in the non-coding region downstream of the stop codon. Overall, the antigenome sequence and the encoded mosaic antigen sequences remained mostly unchanged, demonstrating the genetic stability of the replicating backbone

3.3.3 rBPI3V_{cmut}BVDV vaccine is safe and elicits BVDV-specific antibodies

Intranasal immunization of calves with the rBPI3V_{cmut}BVDV prototype vaccine, a cocktail of five viruses (rBPI3V_{cmut}NS2-5¹⁻²) (Group 1); or with the negative control virus rBPI3V_{cmut}TMSP (Group 3), was well tolerated with no adverse side effects observed following the homologous prime and booster doses (Table 3.2). Similarly, calves immunized subcutaneously with the commercial Bovi-Shield Gold 5 vaccine (Group 2) also exhibited no adverse effects. In all study groups, calves remained healthy throughout the study period, maintained relatively consistent body temperatures, and exhibited steady body weight gain during the immunization phase (Fig. 3.6A-C).

Calves immunized with either the rBPI3V_{cmut}BVDV prototype vaccine or the Bovi-Shield Gold 5 vaccine, seroconverted and isotype switched, and the induced BVDV-specific IgG responses recognized wild-type BVDV-1b (strains California and TGAC) and BVDV-2a (strains A125 and 1373) viruses (Fig. 3.7A). Compared to the negative controls, both vaccine treatment groups

elicited significantly higher BVDV-specific immune responses against all tested BVDV strains (Fig. 3.7B). Notably, calves immunized with the rBPI3V_{cmut}BVDV prototype vaccine elicited higher, but not significant, mean IgG response against two of the tested viruses (BVDV-1b California p=0.4327 and TGAC p=0.9776) compared to the Bovi-Shield Gold 5 MLV vaccinees. While both vaccine treatment groups showed similar mean BVDV-2a A125-specific IgG responses (at 1:1400 serum dilution), one calf in the rBPI3V_{cmut}BVDV vaccinees exhibited exceptionally high responses, detected at 1:4000 serum dilution (Fig. 3.7).

3.3.4 rBPI3V_{cmut}BVDV prototype vaccine elicited cross-neutralizing antibodies

Calves immunized with the rBPI3V_{cmut}BVDV prototype vaccine elicited antibodies that neutralized five BVDV-1 and five BVDV-2 strains, as determined by viral neutralizing assays conducted using sera collected one week after the second boost. Notably, the rBPI3V_{cmut}BVDV prototype vaccine elicited significantly higher mean Viral Neutralizing (VN) titers against BVDV-1b California (CA0401186a) and TGAC strains compared to the Bovi-Shield Gold 5 vaccine (p=0.0031, p=0.0002, respectively) or the negative controls (p=0.0005, p<0.0001, respectively) (Fig. 3.8). Conversely, the Bovi-Shield Gold 5 vaccine elicited higher mean VN titers against BVDV-1a Singer, NADL, and BVDV-1b BJ strains than the rBPI3V_{cmut}BVDV vaccine, with significantly higher titers against NADL (p=0.0153) and BJ (p=0.0492) strains when compared to the negative controls (Fig. 3.8). Notably, VN titers elicited against BVDV-1 Singer, California, NADL, BJ, and TGAC viruses by the rBPI3V_{cmut}BVDV prototype vaccine were 1:128 – 1:256, 1:512 – 1:4096, 1:128 – 1:512, 1:128 – 1:256, and 1:256 – 1:1024, respectively. On the other hand, VN titers elicited by the Bovi-Shield Gold 5 vaccine against the same BVDV-1 viruses were 1:64 – 1:4096, 1:64 – 1:1024, 1:16 – 1:2048, 1:16 – 1:2048, and 1:16 – 1:128, respectively (Fig. 3.8).

Interestingly, the rBPI3V_{mut}BVDV prototype vaccine induced significantly higher mean VN titers against all tested BVDV-2a strains 296NC (p=0.0006), 890 (p=0.0020), 296C (p=0.0464), A125 (p=0.0018), and 1373 (p=0.0025) (Fig. 3.9). Notably, VN titers elicited against all tested BVDV-2a strains 1373, 296NC, 890, 296C and A125, by the rBPI3V_{mut}BVDV prototype vaccine were 1: 512 – 1:4096, 1:512 - 1:4096, 1:256 – 1:4096, 1:128 – 1:2048 and 1:256 – 1:2048, respectively. In comparison, titers elicited by the Bovi-Shield Gold 5 vaccine against the same BVDV-2 viruses were 1:32 – 1:2048, 1:64 – 1:2048, 1:128 – 1:2048, 1:128 – 1:2048 and 1:128 – 2048, respectively (Fig. 3.9).

3.3.5 Post-challenge clinical signs and viremia

Following challenge, mild clinical signs such as decreased appetite, nasal discharge, sluggishness, ocular discharge, and coughing were randomly observed in all treatment groups. All calves developed fever, with mean peak temperatures recorded on day 7 at 102.7°C in the negative control group, day 8 at 103.3°C in the rBPI3V_{mut}BVDV prototype vaccine group, and day 15 at 102.9°C in the Bovi-Shield Gold 5 group (Fig. 3.10A). A slow weight gain was observed in the first week post-challenge in all the treatment groups. By the second week, calves in the rBPI3V_{mut}BVDV group gained an average of 30.8 lbs., increasing from 438 lbs. to 468.8 lbs. Similarly, those in the Bovi-Shield Gold 5 group gained 32 lbs., increasing from 451.8 lbs. to 483.8 lbs. In contrast, calves in the negative control group had the lowest weight gain, increasing by only 25.2 lbs., from 429.8 lbs. to 455 lbs., although this was not statistically significant (Fig. 3.10B).

Interestingly, a decrease in WBC counts was observed in calves immunized with the rBPI3V_{mut}BVDV [from 7,540/ μ l on DPC 0 to 6,520/ μ l on DPC 6] and Bovi-Shield Gold 5 vaccines [from 8,440/ μ l on DPC 0 to 6,920/ μ l on DPC 3], but not in the negative control group.

The greatest reduction in WBC counts was observed in the Bovi-Shield Gold 5 vaccine group on day 3 post-challenge (Fig. 3.10C). This reduction, however, did not translate to leukopenia, given that a leukocyte count below 4,900-5,100/ μ l is considered to cause leukopenia in cows (Roland, Drillich, & Iwersen, 2014). The rBPI3V_{cmut}BVDV vaccinees recovered by day 9 post-challenge, while the Bovi-Shield Gold 5 vaccinees recovered between days 12 and 15 (Fig. 3.10C). All treatment groups also exhibited a decrease in Lymphocyte counts, from 4,200/ μ l on DPC 0 to 3,040/ μ l on DPC 6 in the rBPI3V_{cmut}BVDV vaccinees, from 5,240/ μ l on DPC 0 to 4,100/ μ l on DPC 3 in the Bovi-Shield Gold 5 vaccinees, and from 4,720/ μ l on DPC 0 to 2,920/ μ l on DPC 6 in the negative control group. However, the negative control group had the lowest mean lymphocyte counts throughout most of the challenge phase (Fig. 3.10D). Notably, lymphocyte counts were lower in the Bovi-Shield Gold 5 vaccine group than in the rBPI3V_{cmut}BVDV group for most of the challenge phase. This reduction, however, did not translate to lymphopenia, given that a lymphocyte count below 1,600-2,500/ μ l is considered to cause lymphopenia in cows (Roland et al., 2014). By days 15 to 18 post-challenge, only the rBPI3V_{cmut}BVDV vaccine group had fully recovered (Fig. 3.10D).

Post-challenge, calves immunized with the rBPI3V_{cmut}BVDV prototype vaccine had lower mean BVDV viral load than the Bovi-Shield Gold 5 vaccinees or negative control group. While the BPI3V_{cmut}BVDV vaccinees had lower, but insignificant, mean viremia in blood than the Bovi-Shield Gold 5 vaccinees on day 6 and 15 post-challenge, they showed significantly lower viremia than the negative control group on both day 6 ($p=0.0215$) and day 15 ($p=0.0153$) post-challenge (Fig. 3.11). Notably, by day 15 post-challenge, the lowest viremia titers were observed in 2 out of 5 calves in the rBPI3V_{cmut}BVDV vaccine group and 1 out of 5 calves in the Bovi-Shield Gold 5 vaccinees.

3.3.6 Gross and Histopathologic lesions

Pulmonary lesions were observed in all calves but were generally mild and varied among calves (Fig. 3.12). Bronchopneumonia was characterized by consolidation of up to 80% of the cranial portion of the right or left cranial lung lobe, along with multiple fibrous adhesions between the visceral pleura of the lung with the parietal pleura and mediastinum (Fig. 3.12A(A)). Atelectasis was observed in all groups, but was most significant in the cranioventral lungs, affecting up to 70% of the right middle lung lobe, and the caudal and cranial portions of the right cranial lung lobe, with interlobular emphysema present affecting the right middle lung lobe (Fig. 3.12A(B)). Fibrous or fibrinous pleuritis was also observed in all groups, and was noted along the visceral pleura of the right middle lung lobe and fibrous pleuritis along the visceral pleura of the cranial portion of the right cranial lung lobe (Fig. 3.12A(C)). Other lesions included pericardial effusion, enlarged tracheobronchial lymph nodes, pulmonary edema, and petechial hemorrhage. Notably, of the seven types of gross lesions observed across all three treatment groups, 4 out of 7 gross lesions were observed in the rBPI3V_{Cmut}BVDV vaccinees, and 6 out of 7 lesions were observed in both the Bovi-Shield Gold 5 vaccinees and the negative control rBPI3V_{Cmut}TMSP vaccinees. Overall, the negative control group had more calves (12) with gross lesions than the vaccinated groups (8 calves each) (Fig. 3.12B).

Most calves developed mild to moderate interstitial pneumonia histologically except one calf in the negative control group (Fig. 3.13A-B). Other common histologic lesions were mild to moderate lymphoplasmacytic peribronchiolitis and bronchial or bronchiolar-associated lymphoid tissue hyperplasia. Scoring of the histologic lesions for each group showed no statistically significant difference between the three experimental groups (Fig. 3.13B).

3.4 Discussion

There remains a critical need for safer and more effective vaccines for protection against diverse BVDV strains. Current BVDV vaccines in the United States are based on MLVs and Killed vaccines, which are mainly formulated using representative BVDV-1a and BVDV-2a strains. However, recently identified BVDV-2b and 2c variants in the United States, (Neill et al., 2019) were not neutralized by sera from genotype-2a MLV vaccine (Robert W Fulton et al., 2020). Additionally, although BVDV-1b is the predominant sub-genotype in North America (Robert W Fulton et al., 2020; Neill et al., 2019), nearly all MLVs and KVs are formulated using BVDV-1a. Similar antigenic variation has been observed globally based on viral neutralization tests (Alpay & Yeşilbağ, 2019; Baccili et al., 2019; Grange et al., 2023) or cell-mediated immune responses (Falkenberg et al., 2021), highlighting the limitations of current vaccines in providing broad epitope coverage against diverse circulating BVDV strains. This study aimed to develop a contemporary live vaccine capable of inducing protection against multiple BVDV strains.

Live-vectored vaccines mimic modified live vaccines in that they replicate *in vivo*, eliciting protective BVDV-specific neutralizing antibodies, CD4⁺ T cell, and CD8⁺ cytotoxic lymphocyte responses in calves (Falkenberg, Dassanayake, et al., 2023). Intranasal live vaccines have an added advantage, as they are less vulnerable to interference from maternal-derived antibodies, which can otherwise inhibit effective immunization in young calves. This study utilized a recently described Bovine Parainfluenza-3 virus genotype-C vector (BPI3V_{Cmut}), a replicon capable of retaining its infectivity and immunogenicity (Chapter 2). As such, it elicited neutralizing antibodies against self (rBPI3V_{Cmut}TMSP virus elicited VN titers of 1:64-1:2048 against BPI3Va-c viruses) as well as transgene encoded antigens (Chapter 2). Here, five novel mosaic BVDV antigens, designated E2-NS2-5¹⁻², were designed based on the consensus of more than 200

sequenced BVDV genomes. These antigens were used in generating recombinant BPI3Vc-
vectored viruses (Fig. 3.1), which tested for the potential to protect calves against diverse BVDV
strains following intranasal immunization.

Mosaic BVDV antigenic sequences were designed based on the consensus of all BVDV-
1a, -1b, -2a, -2b, and -2c sequences as of 2019. Specifically, 44, 51, and 112 sequences encoding
E2-NS2-3 of BVDV genotypes -1a, -1b, and -2, respectively, were used, and the resulting mosaic
sequence was designated as E2-NS2-3^{1,2,3} (E2-NS2-3¹⁻³). 77 and 101 sequences encoding NS4-5
of BVDV-1 and -2, respectively, were used, and the resulting mosaic sequence was designated as
NS4-5^{1,2} (NS4-5¹⁻²). These mosaic consensus sequences varied in their conservation levels,
displaying sequence identities of 36.3%, 75.6% and 57.3% to the corresponding E2 protein of
BVDV-1a, -1b and -2, respectively, and 77.9%, 93.2%, and 75.6% identity to the corresponding
NS2-3 protein of BVDV-1a, -1b and -2, respectively. Similarly, mosaic NS4-5¹ and NS4-5²
displayed 72.8% and 70.7% identity to their original protein sequences, respectively (Fig. 3.2).
The varied conservation levels, particularly prominent with the E2^{1a} antigen, which has less than
50% identity to the 44 original sequences, indicate that this strategy incorporated conserved and
variable determinants from the diverse BVDV strains. This approach has potential to provide
broader epitope coverage to improve breadth of protective immunity against disparate strains.
Previous studies have shown that mosaic antigens can prime broad BVDV-specific antibody
repertoire and enhance T cell proliferation, and confer broader protection against BVDV
(Lokhandwala et al., 2017; Mwangi, Fang, Lokhandwala, Bray, & Waghela, 2014; Sangewar et
al., 2020). In this current study, more sequences were used to generate mosaic antigens. It has
previously been shown that immunization with mosaic antigens can induce broader B cell
responses against influenza viruses in mice (Kanekiyo et al., 2019), and confer better protection

against Simian HIV-1 in rhesus monkeys (Barouch et al., 2013) as well as against avian influenza in poultry (Kingstad-Bakke et al., 2019).

The genes encoding mosaic BVDV antigens, E2-NS2-5¹⁻², were used to rescue rBPI3V_{Cmut}E2-NS2-5¹⁻² viruses. Protein expression was authenticated by Immunocytometric analysis and Western blots using tag-specific and gene-specific mAbs (Fig. 3.3), demonstrating that the 2A autocleavable motif successfully enabled the separate expression of the E2¹⁻³ and NS2-3¹⁻³ antigens. The E2 glycoprotein is the major structural protein that interacts with host cell surface receptors (Wang, Deng, Huang, & Chang, 2015) and contains key neutralizing and T-cell epitopes (Deregt, van Rijn, Wiens, & van den Hurk, 1998; Julia F Ridpath, 2013). Several subunit vaccines rely solely on the E2 antigen for protective immunity, including those based on recombinant proteins (Avello et al., 2024; Cheng et al., 2023; Lo, Ryan, Luke, Chang, & Wu, 2023), chimeric constructs (Chowdhury et al., 2021; C.-Y. Liu et al., 2022), virus-like particles (Katsura et al., 2023), DNA vaccines (Sakai et al., 2024), and MHC-II targeted subunit vaccines (Bellido et al., 2021; Bellido et al., 2024; Pecora et al., 2015). In this study, to enhance recognition of the E2¹⁻³ antigens by B cells, its transmembrane and cytoplasmic domains were replaced with those of the BPI3Vc Fusion protein. This modification not only improves packaging of the recombinant virus, but also results in display of the E2 antigen on the surface of infected cells, allowing optimal recognition of both linear and conformational epitopes by B cells (Liang et al., 2016; Julia F Ridpath, 2013). We showed that only the rescued rBPI3V_{Cmut}E2-NS2-3¹⁻³ viruses, but not the cognate rBPI3V_{Cmut}COV19 Spike virus, displayed the E2¹⁻³ ectodomains on the surface of MDBK infected cells, as validated using the mAb 348 against E2 glycoprotein (Fig. 3.4).

The rBPI3V_{Cmut} vector was previously shown to be genetically stable over several rounds of passages, and the presence of ~0.8 and ~3.6 kb transgenes did not affect overall viral titers

(Chapter 2). Similarly, the presence of ~5 kb transgenes did not affect overall viral titers, but displayed a less vigorous attenuated phenotype during the first three days of culture (Fig. 3.5), as observed with other RNA vectored vaccines (Bukreyev, Skiadopoulos, Murphy, & Collins, 2006; Haller, Miller, Mitiku, & Coelingh, 2000). Additionally, the rBPI3V_{cmut} backbone and its encoded transgenes remained relatively stable over nine passages. For the three viruses tested (rBPI3V_{cmut}E2-NS2-3^{1a}, rBPI3V_{cmut}E2-NS2-3^{1b}, and rBPI3V_{cmut}NS4-5²), the only mutations detected after nine rounds of passaging included a L120P mutation in the E2^{1a} gene, a V1030Q mutation in the NS2-3^{1a} gene, a G324S mutation in the F gene of the rBPI3V_{cmut}NS4-5² virus and some mutations (I25S, I28S, M27T, L39P, V1261A) in the NS4-5² gene. Notably, some mutations were observed in the P2 progenies but not the P9 progenies, suggesting that the P2 progenies were not clonal (Table 3.1), suggesting that it is important to conduct plaque purifications to generate clonal virus populations prior to conducting *in vivo* studies.

Immunization of five-month-old Holstein calves with either the rBPI3V_{cmut}BVDV prototype vaccine or the commercial Bovi-Shield Gold 5 vaccine elicited BVDV-1 and -2 specific antibody responses (Fig. 3.7). Vaccine-induced neutralizing antibodies are essential for protecting young calves against BVDV infections (Brar et al., 1978; Chamorro et al., 2015). Notably, the rBPI3V_{cmut}BVDV prototype vaccine induced a broader neutralization breadth than the commercial Bovi-Shield Gold 5 vaccine. Calves immunized with the rBPI3V_{cmut}BVDV vaccine had significantly higher VN titers against BVDV-1b California and TGAC (p=0.0031 and p=0.0002, respectively). Furthermore, compared to the negative control group, the rBPI3V_{cmut}BVDV prototype vaccine, but not the Bovi-Shield Gold 5 vaccine, elicited significantly higher VN titers against 1b California (p=0.005), TGAC (p<0.0001) and all BVDV-2a 1373 (p=0.0025, 296NC (p=0.006), 890 (p=0.002), 296C (p=0.0464), and A125 (p=0.0018) viruses (Fig. 3.8 and 9). This

demonstrated that the rBPI3V_{cmut}BVDV prototype vaccine elicited more potent VN titers against both the most prevalent BVDV-1b genotype in the United States (Falkenberg, Ma, et al., 2023; Robert W Fulton, 2015; Walz et al., 2020) and all the tested BVDV-2 strains. Since VN titers of 1:128 are considered protective against BVDV (Robert W Fulton et al., 2020), our findings show that the rBPI3V_{cmut}BVDV vaccine elicited protective titers in nearly all calves compared to the Bovi-Shield Gold 5 vaccine under the same immunization schedule and conditions. Interestingly, although the vaccine strains in the commercial Bovi-Shield Gold 5 are BVDV-1a strain NADL and BVDV-2a strain 53637 (Falkenberg et al., 2021), all calves immunized with the rBPI3V_{cmut}BVDV prototype vaccine had VN titers of more than 1:128 against BVDV NADL. In contrast, one calf in the commercial vaccine group had very low titers of 1:16 against BVDV NADL. Previous studies have shown that recombinant adenoviruses expressing mosaic BVDV antigens (Lokhandwala et al., 2017), purified recombinant E2 antigens (Sangewar et al., 2020), or a combination MLV vaccine (Stephanie Perkins-Oines et al., 2023) elicited higher neutralizing antibody titers against BVDV-1 strains. In contrast, the rBPI3V_{cmut}BVDV prototype vaccine elicited higher VN titers against BVDV-2 strains and against the BVDV-1b strains California and TGAC. The bias towards BVDV-2 neutralization was likely due to the higher number of BVDV-2 sequences used in designing the mosaic polypeptides, suggesting an increased number of conserved BVDV-2 epitopes. While the duration of immunity conferred by the Bovi-Shield Gold 5 vaccine is approximately nine months (Zoetis, 2025), the duration of immunity conferred by the rBPI3V_{cmut}BVDV prototype vaccine is yet to be empirically determined. Although we did not quantify the levels of mucosal antibodies, other recent studies show that intranasal, but not intramuscular, administration of purified E2Fc subunit vaccines results in an increase in the production of neutralizing mucosal antibodies (Cheng et al., 2023).

Acute BVDV infection typically causes different clinical signs such as fever, diarrhea, nasal discharge, coughing, loss of appetite, and ocular discharges along with hematologic changes such as leukopenia and lymphopenia that can lead to immunosuppression (Campbell, 2004; Loneragan, 2005). Findings from this study indicate that clinical BVDV disease was mild, and no significant differences were observed between the vaccine treatment groups, which was consistent with previous vaccine efficacy studies (Bittar et al., 2020; Chamorro et al., 2015; Masic et al., 2021; Taberner et al., 2024). The observed increase in temperature towards the end of the study in the calves immunized with the commercial vaccine was due to one calf (ID 5657) that fell sick due to unrelated conditions (Fig. 3.10A). Weight gain affects the economic impact of beef animals. During the challenge phase, vaccinated calves exhibited higher weight gain compared to the non-vaccinated controls (Fig. 3.10B), similar to other challenge studies showing significantly higher carcass weight in vaccinated animals (Chamorro et al., 2015; Tapiolas et al., 2024). Interestingly, while all immunized calves exhibited a decrease in white blood cell count, while the non-vaccinated negative control calves did not. Although all treatment groups exhibited a decrease in lymphocyte counts, the greatest decrease was observed in the negative control group, followed by the Bovi-Shield Gold 5 vaccine group (Fig. 3.10C and D). Importantly, the observed decrease in this study was not below the 4,900-5,100/ μ l threshold considered to cause leukopenia or the 1,600-2,500/ μ l threshold considered to cause lymphopenia in cows (Roland et al., 2014). A recent study reported that calves immunized intranasally, but not parenterally, with a BVDV MLV vaccine in the face of maternal antibodies, developed leukopenia upon challenge (Stephanie Perkins-Oines et al., 2023; S Perkins-Oines et al., 2023). However, we observed decreased WBC counts in both the intranasally immunized rBPI3V_{Cmut}BVDV and parenterally immunized Bovi-Shield Gold 5 groups. It is possible that administering three immunization doses, three weeks apart (despite Bovi-

Shield Gold 5 being recommended for re-vaccination at least after nine months or annually (Zoetis, 2025), may have induced a stronger inflammatory response upon challenge. This may have led to a cytokine surge and lymphocyte margination causing leukocytes to move from blood stream into tissues, leading to transient decrease in WBC counts (Beyer et al., 2002; Yamamoto et al., 2006), as supported by data showing the presence of more lymphocytes in the vaccinated groups than the negative control group (Fig. 3.10D). Following challenge, calves immunized with the rBPI3V_{cmut}BVDV prototype vaccine had lower levels of viremia than calves immunized with the Bovi-Shield Gold 5 vaccine and the negative control calves on day 6 ($p=0.0215$) and day 15 ($p=0.0153$) post-challenge (Fig. 3.11). This correlated with the significantly higher serum antibody and highest VN titers against BVDV California elicited by the rBPI3V_{cmut}BVDV vaccine. Our findings are similar to most studies showing that effective vaccination reduces viremia following challenge (Bellido et al., 2021; Bellido et al., 2024; Lokhandwala et al., 2017; Sangewar et al., 2020; Taberner et al., 2024). The gross and histopathologic lesions observed in this study were mainly consistent with those reported in other BVDV vaccine efficacy studies (Chowdhury et al., 2021; Gómez-Romero et al., 2023; C.-Y. Liu et al., 2022; Stephanie Perkins-Oines et al., 2023). Notably, of the seven specific types of gross lesions observed, only 4 out of 7 were observed in the rBPI3V_{cmut}BVDV vaccinees. Bronchopneumonia has been identified as a gross diagnostic lesion following BVDV infection (Caswell, Hewson, Slavić, DeLay, & Bateman, 2012; Chowdhury et al., 2021). We observed bronchopneumonia in one calf immunized with the Bovi-Shield Gold 5 vaccine (5623), which was confirmed by the histopathologic assessment. Overall, more non-vaccinated negative control calves exhibited gross lesions (Fig. 3.12). The most common histologic lesions were interstitial pneumonia, mild to moderate lymphoplasmacytic peribronchiolitis, and bronchial or bronchiolar-associated lymphoid tissue hyperplasia (Fig. 3.13).

It is yet to be empirically determined whether the higher histologic lung lesion scores observed in BVDV-immunized groups is related to the immunogen dosing, administered three times, three weeks apart.

Overall, the data presented here support use of the BPI3Vc_{mut} vector for development of broadly protective BVDV vaccines. Neutralizing antibodies block initial viral infection, while cell-mediated immune responses facilitate the clearance of viral-infected cells (Beer et al., 1997; Howard et al., 1992). Neonates and yearlings are particularly susceptible to infection in the presence of PI calves; therefore, measures taken to prevent that initial infection, provided by potent neutralizing antibodies, are indispensable. Several live attenuated or subunit vaccines have recently been evaluated for efficacy in protecting cattle against BVDV or potential use as vaccine candidates, such as a DIVENCE vaccine for fetal and feedlot protection (Taberner et al., 2024; Tapiolas et al., 2024) that also had neutralizing antibodies in milk samples (Montbrau et al., 2024), a commercial combination MLV in the United States (Masic et al., 2021), other MLVs (Abd El Fadeel et al., 2022; Palomares et al., 2021) and other subunit vaccines, including a MHC-II targeted subunit vaccine already tested in feedlot cattle (Avello et al., 2024; Bellido et al., 2021; Bellido et al., 2024; Cheng et al., 2023; Gómez-Romero et al., 2023). However, these are single-strain-based vaccines, and, given the diverse nature of BVDV, recent data show that single-strain-based vaccines are poor in cross-neutralizing diverse BVDV strains. For instance, a commercial MLV Mucosiffa vaccine containing BVDV-1a vaccine strain elicited lower titers against circulating European BVDV-1f, -1e, and -2a field strains (Grange et al., 2023). A commercial polyvalent MLV vaccine elicited lower protective titers against Turkish BVDV-1b, -2b, and -1r field strains (Alpay & Yeşilbağ, 2019). BVDV-1 and -2 inactivated vaccines did not elicit neutralizing antibodies against the BVDV-2 (VS253) strain (70). Therefore, data from this study showed that rationally

designed mosaic antigens, derived from the consensus of more than 200 sequenced BVDV genomes, broadly neutralized diverse BVDV strains, especially BVDV-2 strains and the most predominant BVDV-1b genotype in the United States. It is worth noting that although we did not test neutralization against BVDV-2b and -2c strains, the design of our mosaic antigens included BVDV-2b and -2c sequences. Therefore, it is possible to achieve protective VN titers against these emergent strains.

In conclusion, results from this study show that five novel mosaic BVDV E2-NS2-5¹⁻² antigens designed using protein sequences from BVDV-1 and -2 genomes were generated, and the rBPI3V_{Cmut}E2-NS2-5¹⁻² viruses rescued displayed the E2 antigens on the surface of infected cells for optimal recognition by B cells. Intranasal immunization with the rBPI3V_{Cmut}BVDV virus cocktail elicited broadly neutralizing antibodies that significantly reduced viremia following challenge with wild-type virus. This pilot study opens a platform for addressing BVDV antigenic variability in future vaccine developments, given the versatility of upgrading antigenic determinants and the ease of cloning into the BPI3Vc backbone. Further refinement, such as optimizing the dosing regimen and analyzing the cell-mediated immune profile, is needed to achieve more robust and lasting immunity in cattle.

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Ethics statement

Animal study was conducted in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University Institutional Animal Care and Use Committees (IACUC registration # 4825) and Institutional Biosafety Committee (IBC registration # 1684).

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Disclosures

Authors have no financial conflict of interest to disclose.

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Table 3.1. Sequencing rBPI3Vc_{mut}E2-NS2-5 viral RNA at passage 2 and passage 9

Virus	Passage	Gene	Identity Percentage	Read Coverage	Genomic Coverage
rBPI3Vc _{mut} E2-NS2-3 ^{1a}	2	<i>N</i>	100	14446.03787	100
		E2-NS2-3 ^{1a}	98.62804878	1.762729124	7.484725051
		<i>P</i>	100	10270.99242	100
		<i>M</i>	100	51900.45915	100
		<i>F_HN</i>	100	3719.36342	100
		<i>L</i>	99.985283296541581	8155.080059	100
	9	<i>N</i>	100	963.9187843	99.85052317
		E2-NS2-3 ^{1a}	98.5757884	2.345723014	48.62525458
		<i>P</i>	100	397.875	99.33712121
		<i>M</i>	100	50133.61768	99.93902439
		<i>F_HN</i>	100	178.2311956	99.47215624
		<i>L</i>	99.985283296541581	292.5557027	99.85283297
rBPI3Vc _{mut} E2-NS2-3 ^{1b}	2	<i>N</i>	100	11506.45042	100
		E2-E2-NS2-3 ^{1b}	99.88256019	2388.156782	100
		<i>P</i>	100	7425.342803	100
		<i>M</i>	100	49388.64024	100
		<i>F_HN</i>	99.973607812087621	2696.85801	100
		<i>L</i>	99.985283296541581	7157.233848	99.9852833
	9	<i>N</i>	100	13646.7378	99.93902439
		E2-E2-NS2-3 ^{1b}	99.88267501	1418.781953	100
		<i>P</i>	100	2606.412556	99.90034878
		<i>M</i>	100	940.2556818	99.71590909
		<i>F_HN</i>	100	411.7487464	99.94721562
		<i>L</i>	99.985283296541581	1044.572627	99.9852833
rBPI3Vc _{mut} NS4-5 ²	2	<i>N</i>	100	11656.61236	100
		NS4-5 ²	100	3030.389363	100
		<i>P</i>	100	8472.04072	100
		<i>M</i>	100	2729.286355	100
		<i>F_HN</i>	99.973607812087621	53906.25366	100
		<i>L</i>	99.985283296541581	5114.187638	99.9852833
	9	<i>N</i>	100	16308.4061	99.87804878
		NS4-5 ²	99.13201453	3670.629111	100
		<i>P</i>	100	3658.872945	99.95017439
		<i>M</i>	100	1454.851326	99.62121212
		<i>F_HN</i>	99.947215624175243	948.4283452	99.94721562
		<i>L</i>	99.985283296541581	2602.053127	99.9852833

Table 3.2. BVDV Calf Immunization protocol

Group	ID	Immunogen	Route	Prime-boost dose/calf
1. rBPI3V_{Cmut}BVDV	5673	<i>rBPI3V_{Cmut}BVDV cocktail viruses:</i>	IN	4.4 x10 ⁹ TCID ₅₀ , 4.4 x10 ⁹ TCID ₅₀ , 8.4 x10 ¹⁰ TCID ₅₀
	5694	rBPI3V _{Cmut} BVDV E2-NS2-3 ^{1a} , E2-NS2-3 ^{1b}		
	5715	E2-NS2-3 ² , NS4-5 ¹ , NS4-5 ² viruses		
	5717			
	5725			
2. Bovi-Shield Gold 5 (Zoetis)	5656	<i>Commercial MLV cocktail:</i>	SQ	2 ml, 2 ml, 2ml
	5657	Infectious bovine rhinotracheitis		
	5688	Bovine viral diarrhea virus 1 and 2		
	5698	Parainfluenza 3 virus		
	5723	Bovine respiratory syncytial virus		
3. rBPI3V_{Cmut}TMSP	5663		IN	1.4 x10 ⁹ TCID ₅₀ , 2.9 x 10 ⁹ TCID ₅₀ , 1.2 x10 ¹⁰ TCID ₅₀
	5696	rBPI3V _{Cmut} TMSP virus		
	5710			
	5719			
	5724			

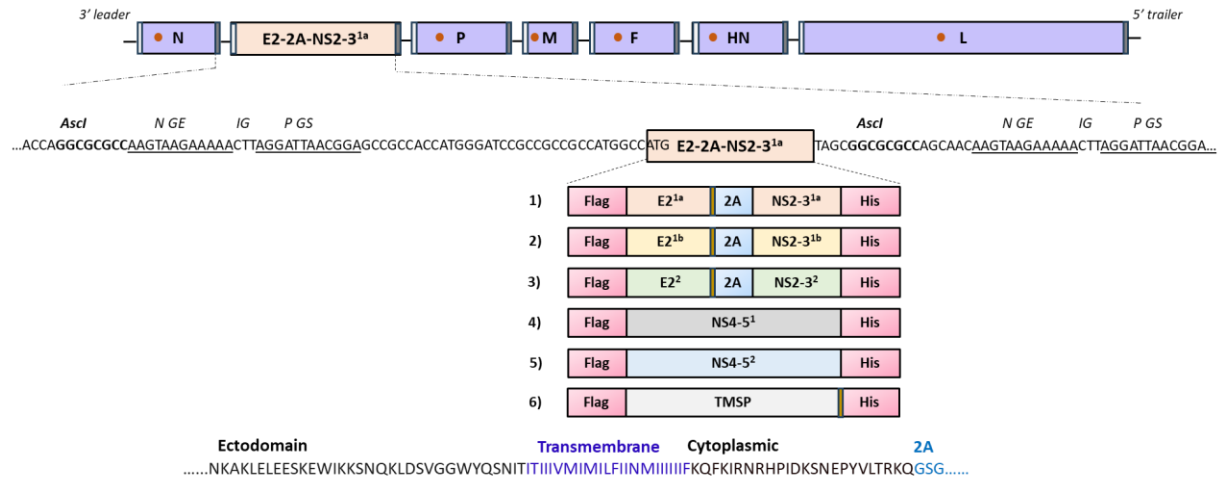
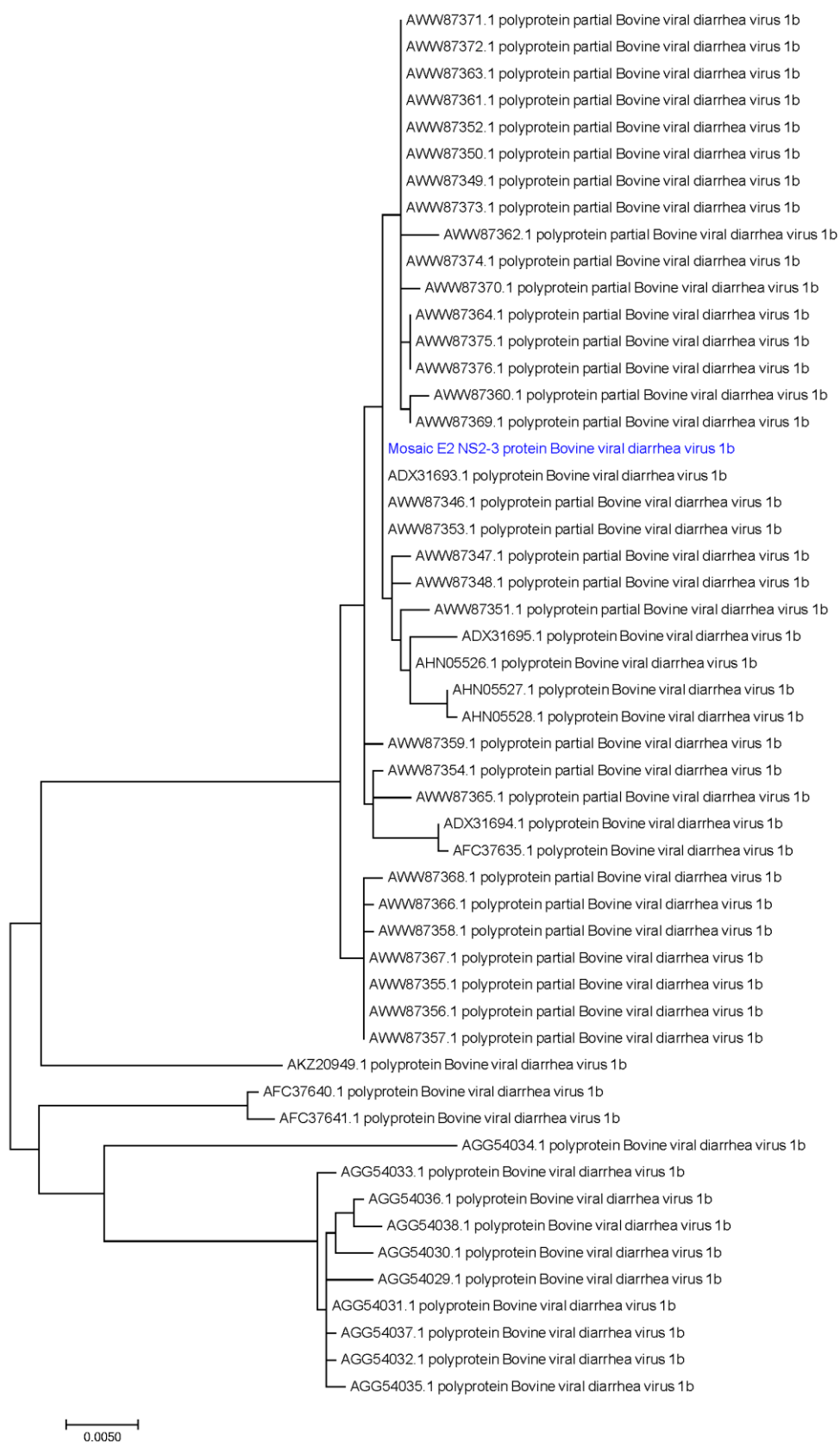


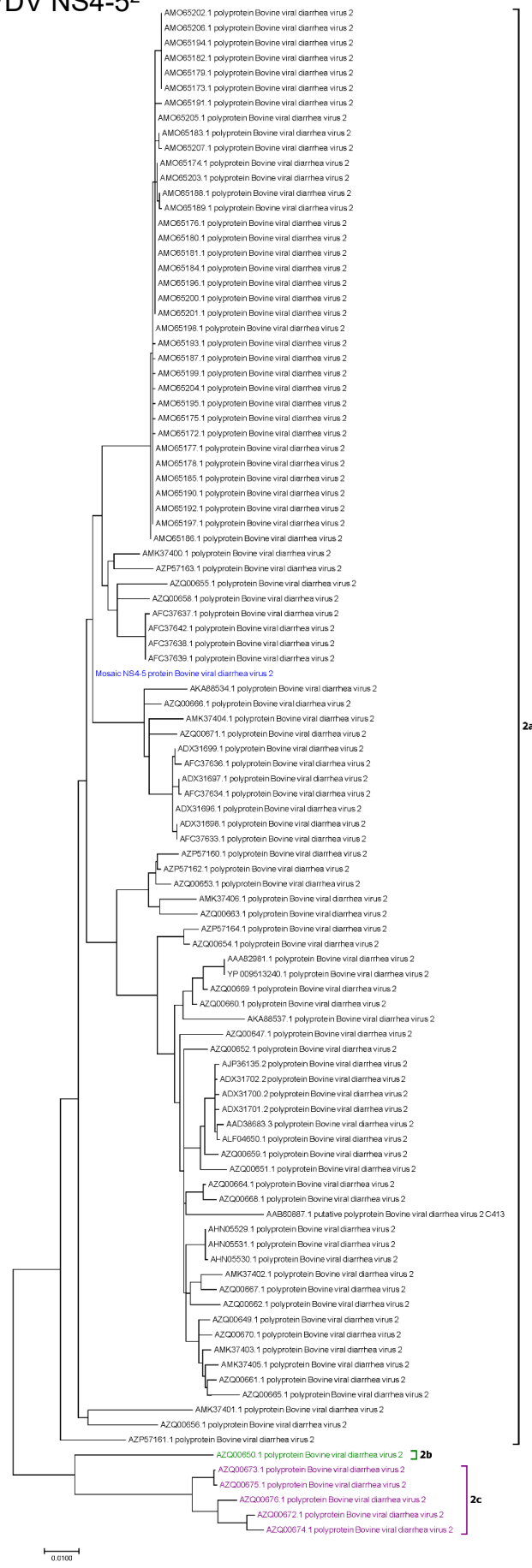
Figure 3.1. Attenuated BPI3Vc expression constructs encoding BVDV antigens.

Design of the BPI3Vc constructs encoding mosaic BVDV 1a, 1b, and 2 antigenic cassettes: 1) E2-NS2-3^{1a}; 2) E2-NS2-3^{1b}; 3) E2-NS2-3²; 4) NS4-5¹; and 5) NS4-5². 6) TMSP represents a negative control antigen cassette. Transgenes are flanked by gene start (white bar) and gene end (grey bar) transcription signals, and cloned, in-frame, between the genes encoding N and P. All genes are separated by an intergenic trinucleotide sequence (CTT), for expression as separate mRNA. E2-NS2-3¹⁻² antigenic cassettes were designed for multicistronic expression separated by an auto-cleavable domain, 2A. The E2¹⁻² and TMSP cassettes contain transmembrane and cytoplasmic domains of the BPI3Vc fusion protein. Flag tag and His tag genes are added in-frame at the N' end and C' end, respectively, to assess recombinant protein expression.

A) BVDV E2-NS2-3^{1b}



B) BVDV NS4-5²



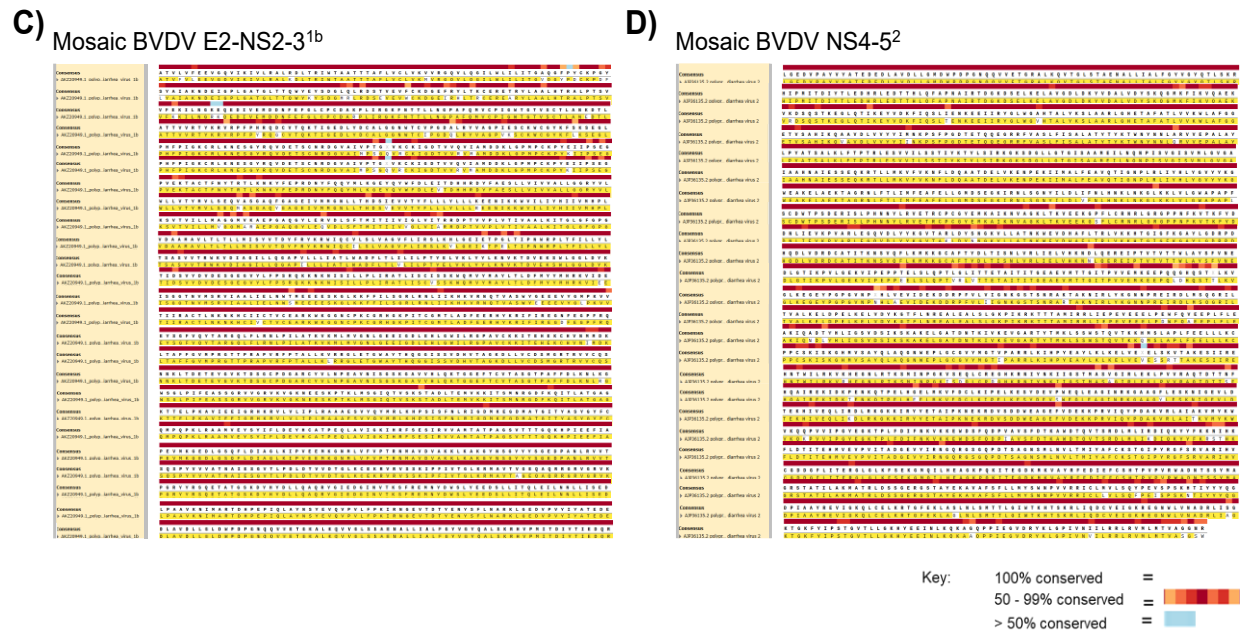


Figure 3.2. Design of mosaic BVDV antigens.

Maximum likelihood phylogenetic tree showing clustering of the consensus E2-NS2-3^{1b} sequence **(A)** and NS4-5² sequence **(B)** (shown in blue) derived from 51 BVDV E2-NS2-3^{1b} and 101 BVDV NS4-5² polypeptide sequences, respectively. Level of conservation of mosaic E2-NS2-3^{1b} **(C)** and NS4-5² **(D)** polypeptide sequences, respectively.

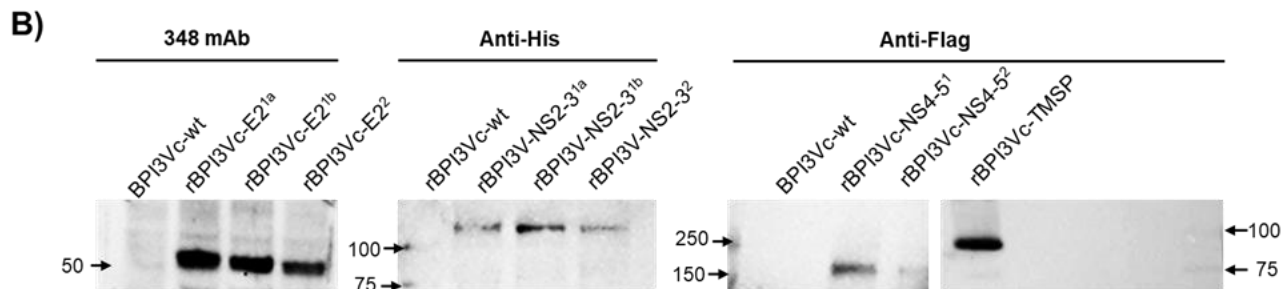
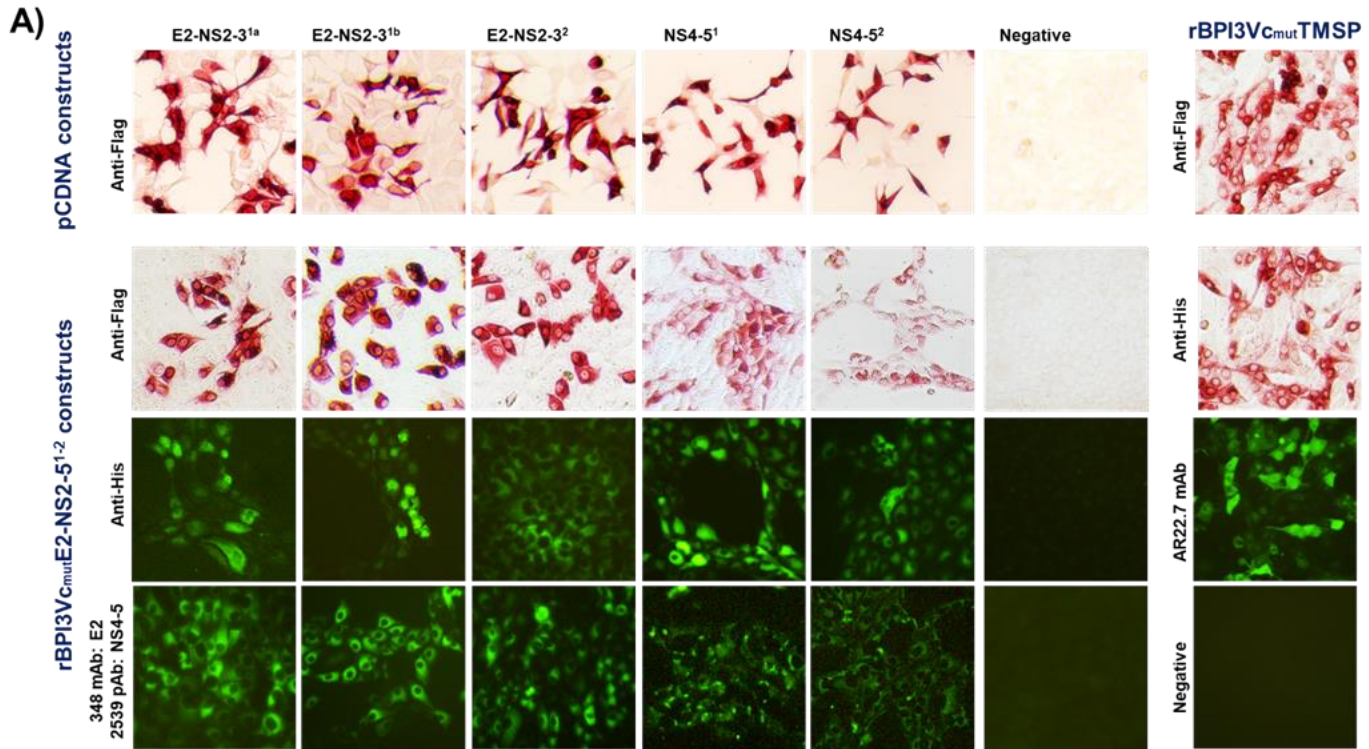


Figure 3.3. Mosaic BVDV antigens protein expression.

A) Top panel: Protein expression by the pCDNA constructs encoding mosaic BVDV E2-NS2-3^{1a}, 1b and 2 and NS4-5¹ and 2 was confirmed by immunocytometric analysis using anti-Flag monoclonal antibody (mAb). Bottom panel: Protein expression by the rescued rBPI3Vc_{mut} E2-NS2-5¹⁻² and rBPI3Vc_{mut}TMSP viruses was similarly confirmed as above, but by using: i) Anti-Flag mAb to detect E2^{1a}, 1b, and 2; ii) Anti-His mAb to detect NS2-3^{1a}, 1b, and 2; iii) mAb 348 to detect BVDV 1 and 2 E2 glycoprotein; iv) Anti-Flag mAb to detect Flag-tag located at the N-terminal of NS4-5¹⁻² anti-His mAb to detect His-tag located at the C-terminal or anti-BVDV polyclonal antibody (pAb). Expression of TMSP was confirmed by using the following mAbs: anti-Flag, anti-His, and TMSP-specific mAb 22.7. **B)** Protein expression was also confirmed by Western Blot analysis using mAbs 348, anti-His, and anti-Flag.

Anti-E2 mAb 348

Anti-TMSP mAb AR22.7

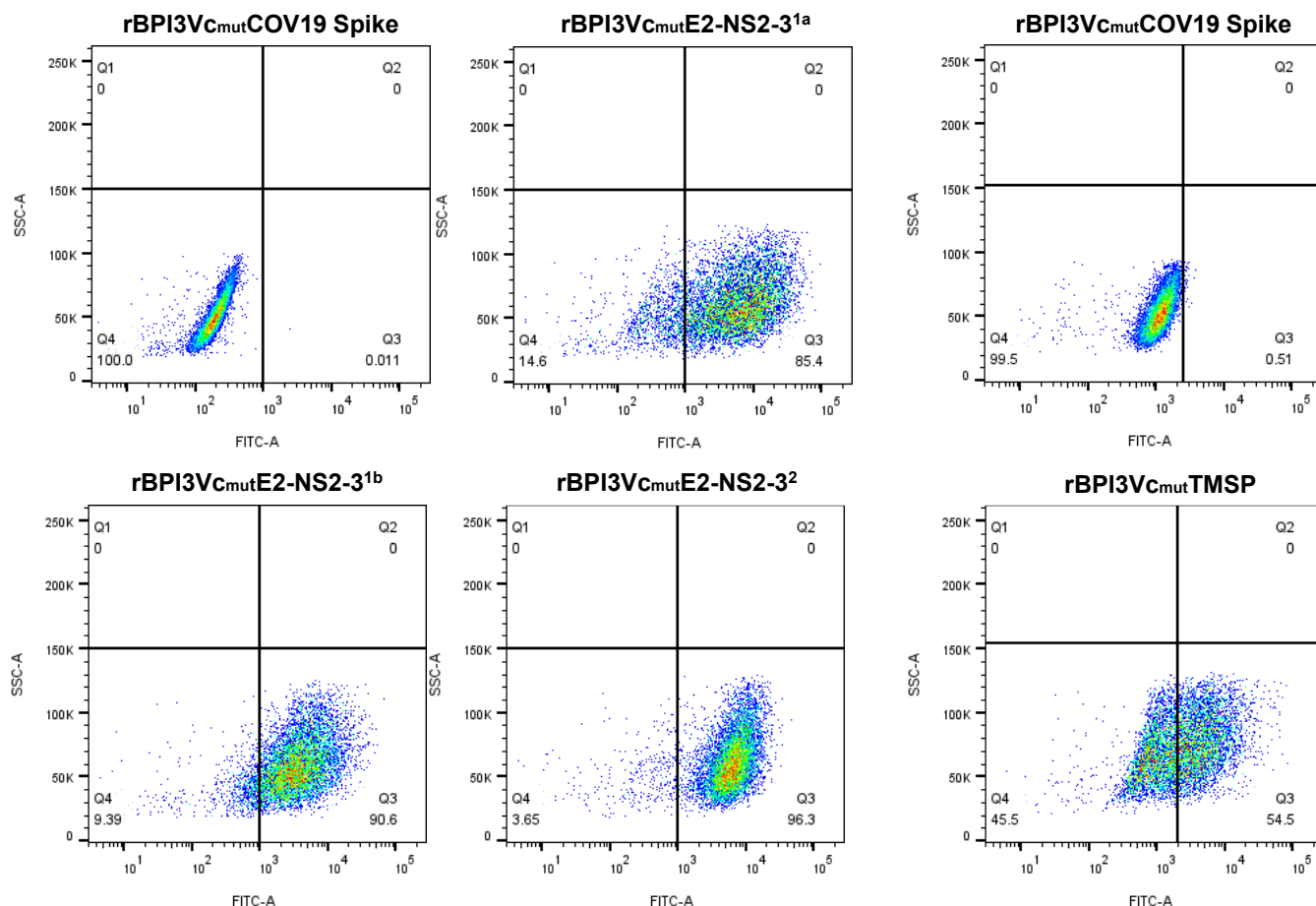


Figure 3.4. Surface display of mosaic BPI3V E2¹⁻² antigens.

Rescued rBPI3V_{cmut}E2-NS2-3^{1a, 1b, 2} and rBPI3V_{cmut}TMSP viruses, but not the rBPI3V_{cmut}Spike virus, displayed the E2^{1a}, E2^{1b}, E2², and the TMSP antigens, respectively, on the surface of infected MDBK cells as confirmed using the E2-specific mAb 348 or the TMSP-specific mAb AR22.7, respectively.

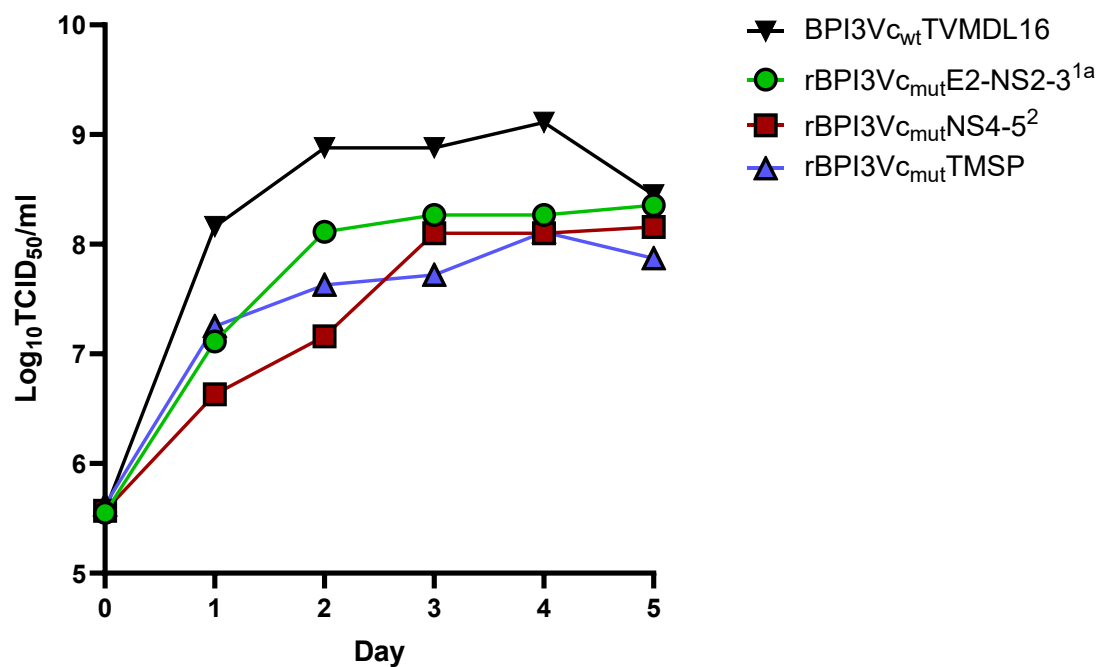


Figure 3.5. Multicycle replication growth curve.

Replication growth curves in MDBK cells infected at an MOI of 1 with either BPI3V_{cwt}TVMDL16 wild-type virus, rBPI3V_{cmut}E2-NS2-3^{1a}, rBPI3V_{cmut}NS4-5², or rBPI3V_{cmut}TMSP virus and incubated at 37°C for four days. Virus aliquots were sampled every 24 hours and quantified by TCID₅₀ virus titration.

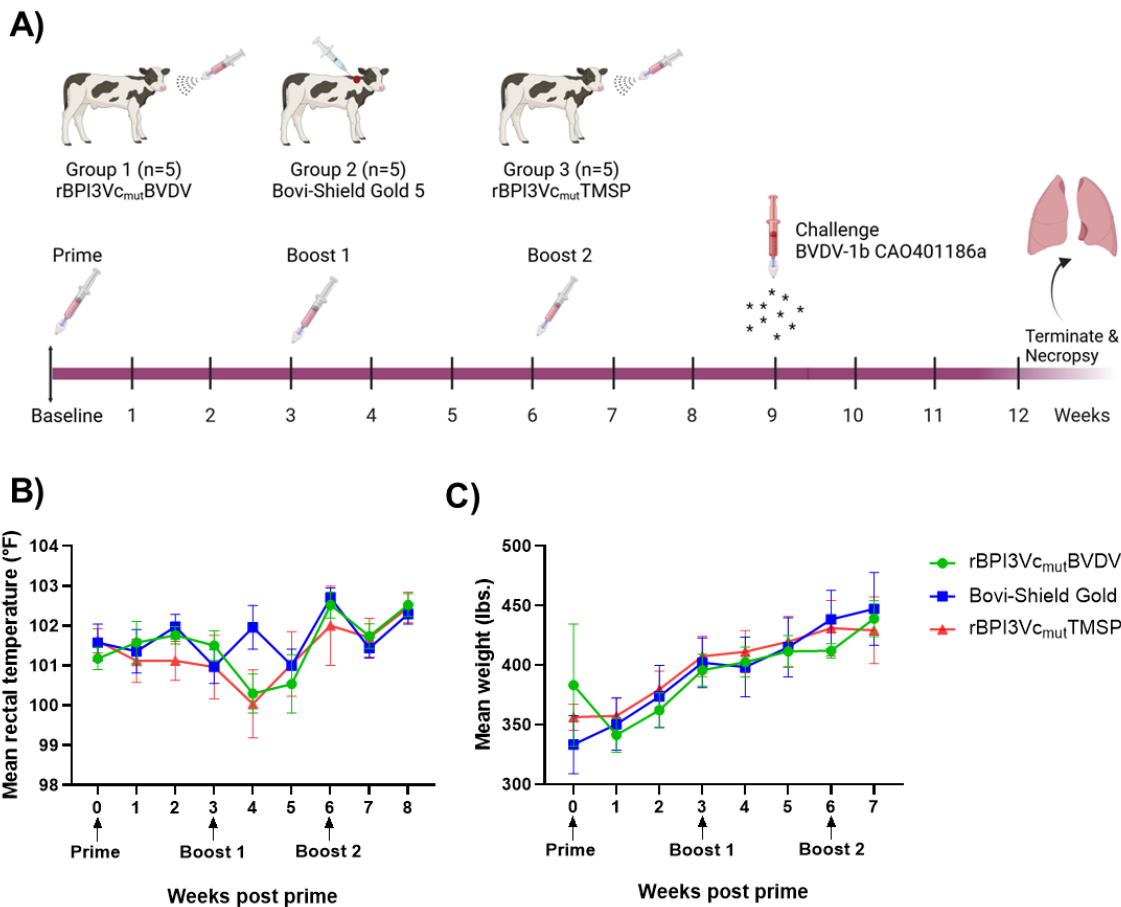


Figure 3.6. Animal study.

A) Immunization design and timeline plan. Calves were assigned to three treatment groups: 1) rBPI3Vc_{mut}BVDV vaccine; 2) Bovi-Shield Gold 5 BVDV commercial vaccine; and 3) rBPI3Vc_{mut}TMSP control virus. After one week of acclimatization, calves in Groups 1 and 3 received an intranasal prime while calves in Group 2 received a subcutaneous priming dose, followed by booster doses at three-week intervals. Nine weeks post-priming, all calves were challenged via intranasal administration of BVDV Type 1b strain California (CA0401186a). The study was terminated at week 13. Weekly sampling for whole blood, serum and nasal swabs was conducted. B) Mean rectal temperature. C) Mean weight gain for each group measured every week

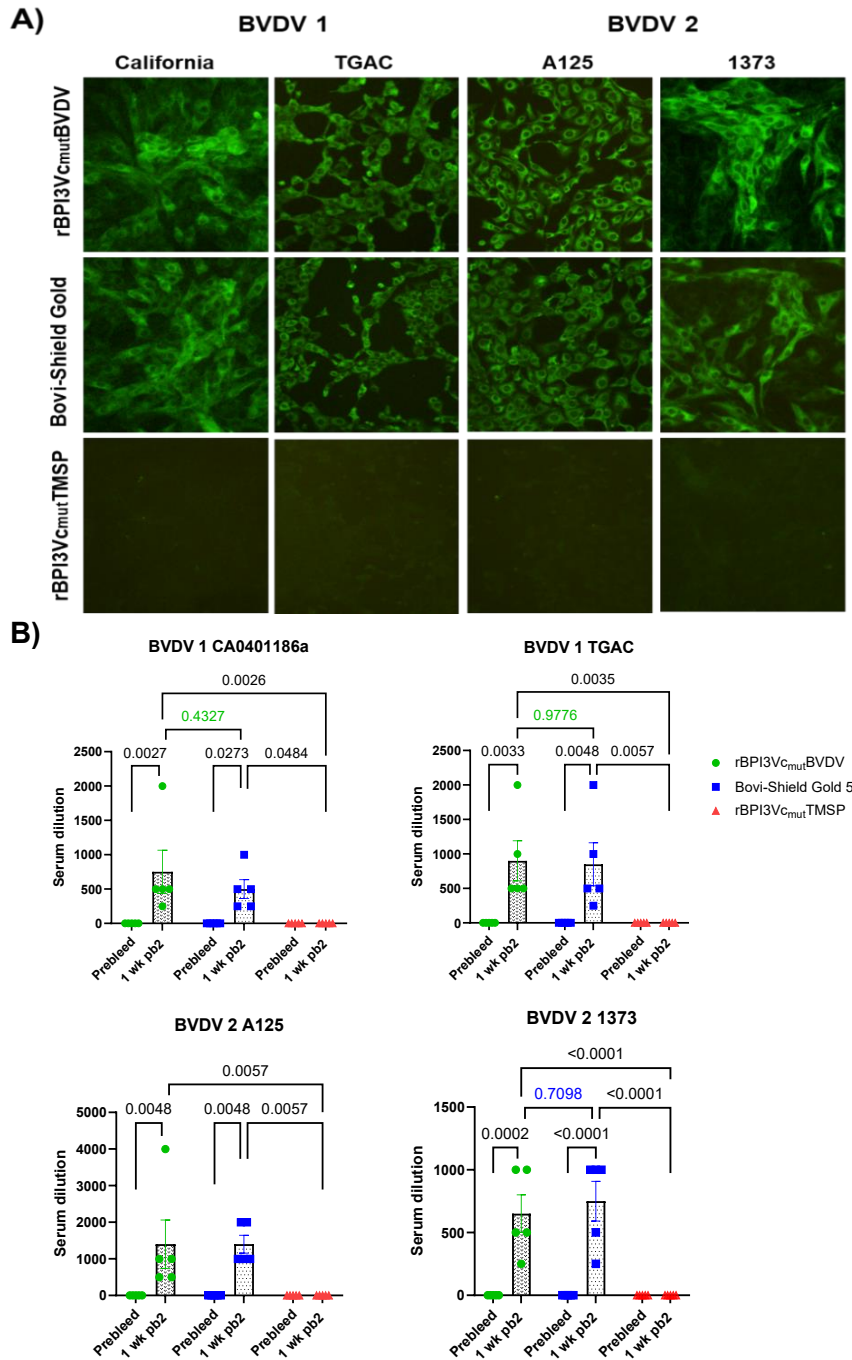


Figure 3.7. Antibody response.

Serum antibodies recognized wild-type BVDV 1 California (CA011401186a), TGAC and BVDV 2 A125, and 1373 viruses as evaluated by Immunofluorescence staining (A) and endpoint dilution staining using sera collected before immunization (Prebleed) and one week post-boost 2 (1 wk pb2) (B). Bars represent group means and statistical differences in group means.

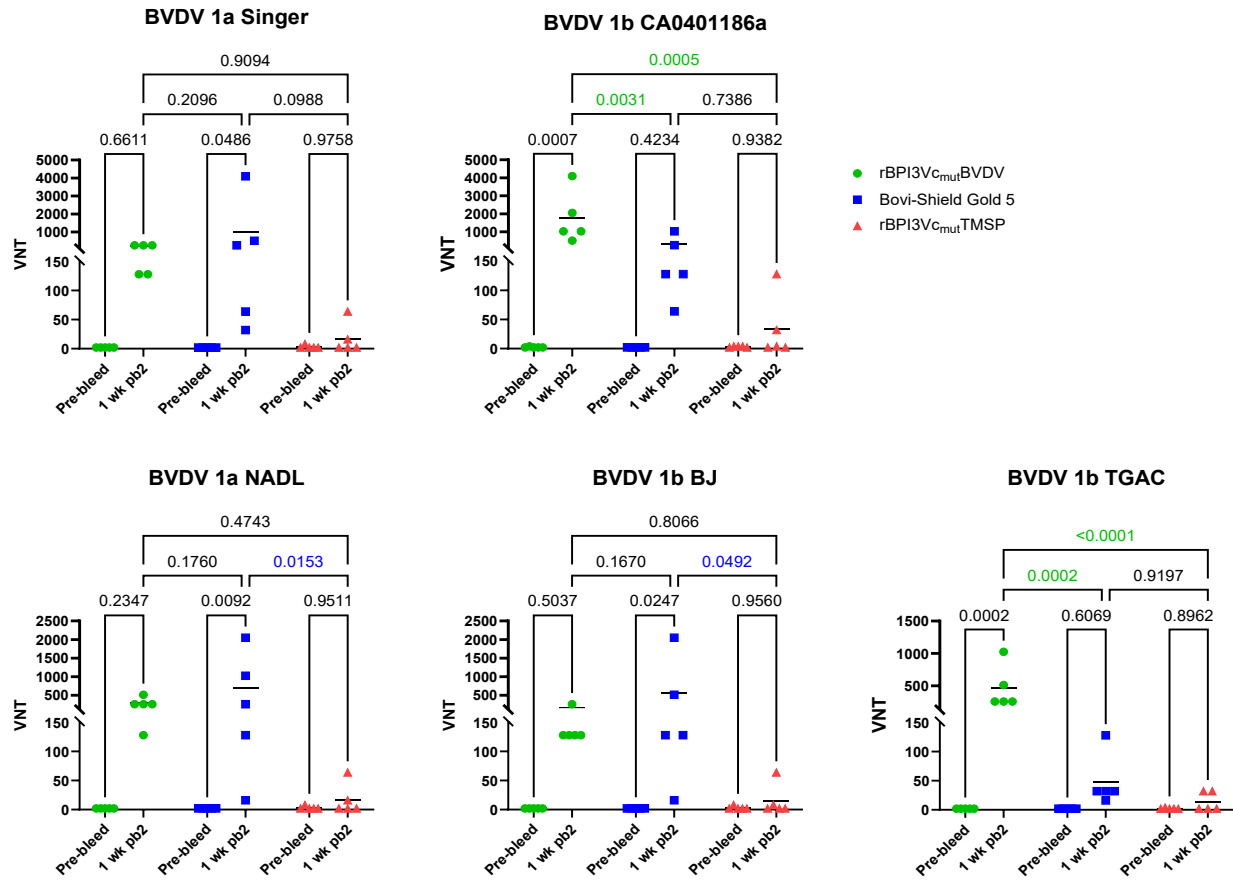


Figure 3.8. BVDV-1-specific neutralizing antibody titers.

BVDV-1-specific neutralization titers in calves immunized with either the rBPI3Vc_{mut}BVDV vaccine, Bovi-Shield Gold 5 vaccine, or the rBPI3Vc_{mut}TMSP control virus construct were determined by virus neutralization assays against the representative BVDV-1 strains using sera collected before immunization (Prebleed) and one-week post-boost-2. Bars represent mean group titers and statistical differences in group means.

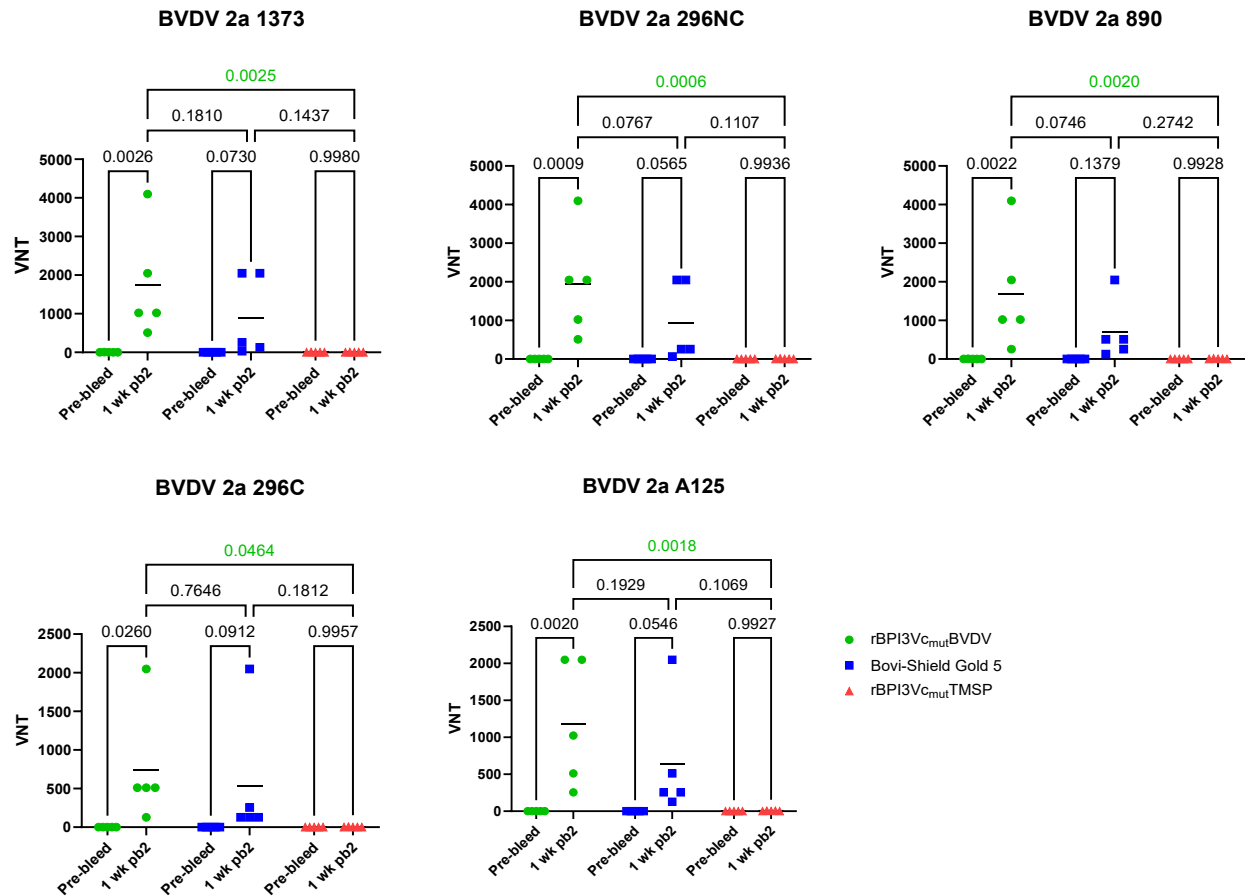


Figure 3.9. BVDV-2-specific neutralizing antibody titers.

BVDV-2-specific neutralization titers in the calves immunized with either the rBPI3Vc_{mut}BVDV vaccine, Bovi-Shield Gold 5 vaccine, or the rBPI3Vc_{mut}TMSP control virus construct were determined by virus neutralization assays against the representative BVDV-2 strains using sera collected before immunization (Prebleed) and one-week post-boost-2. Bars represent mean group titers and statistical differences in group means.

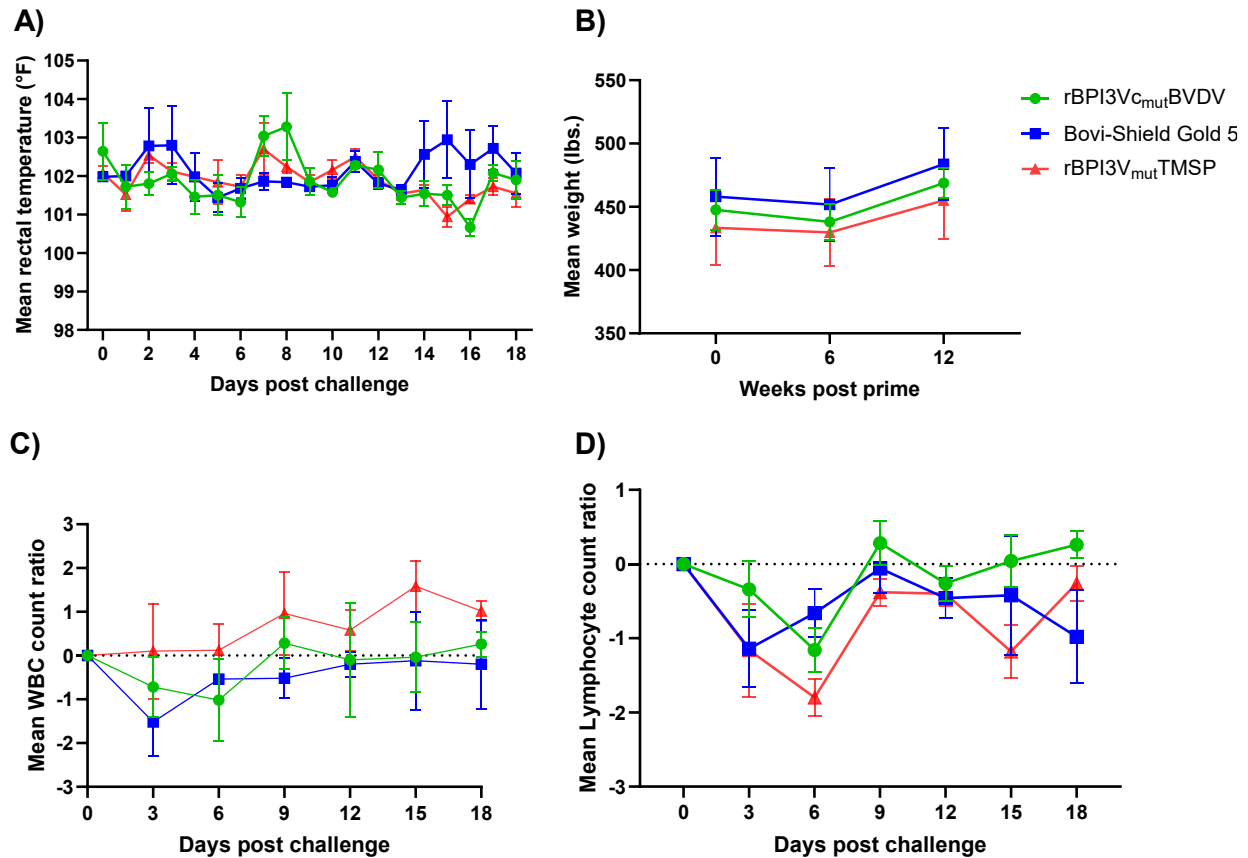


Figure 3.10. Clinical outcomes post-challenge.

A) Mean rectal temperatures recorded daily for each group is plotted; and **B)** mean gain in body weights recorded on day of challenge, six- and twelve-days post-challenge for each group is plotted. Mean change in **C)** white blood cell; and **D)** lymphocyte count ratios for each group quantified on every third day post-challenge is plotted.

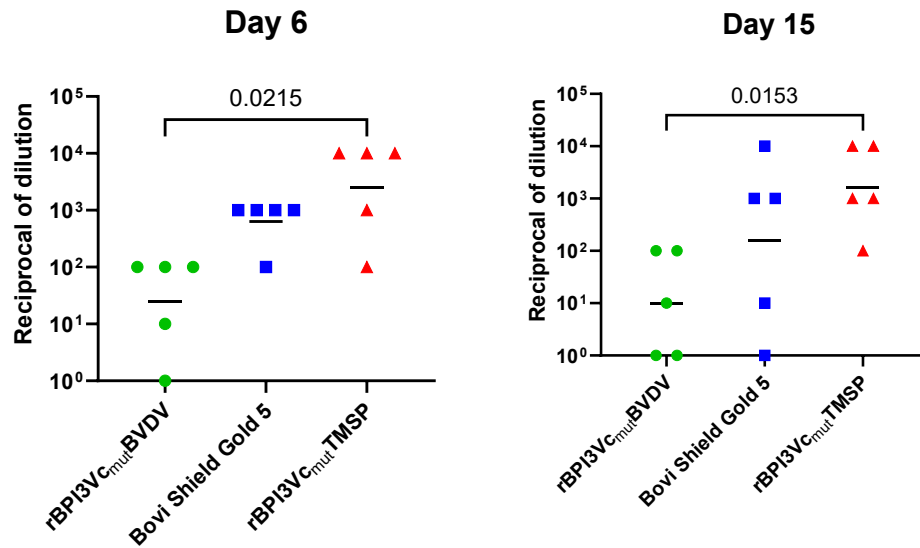


Figure 3.11. Viremia in calves post-challenge.

Viremia was determined in blood samples collected on day 6 (A) and day 15 (B) post-challenge with BVDV-1b strain CA0401186a. Mean group dilutions are represented by the bars.

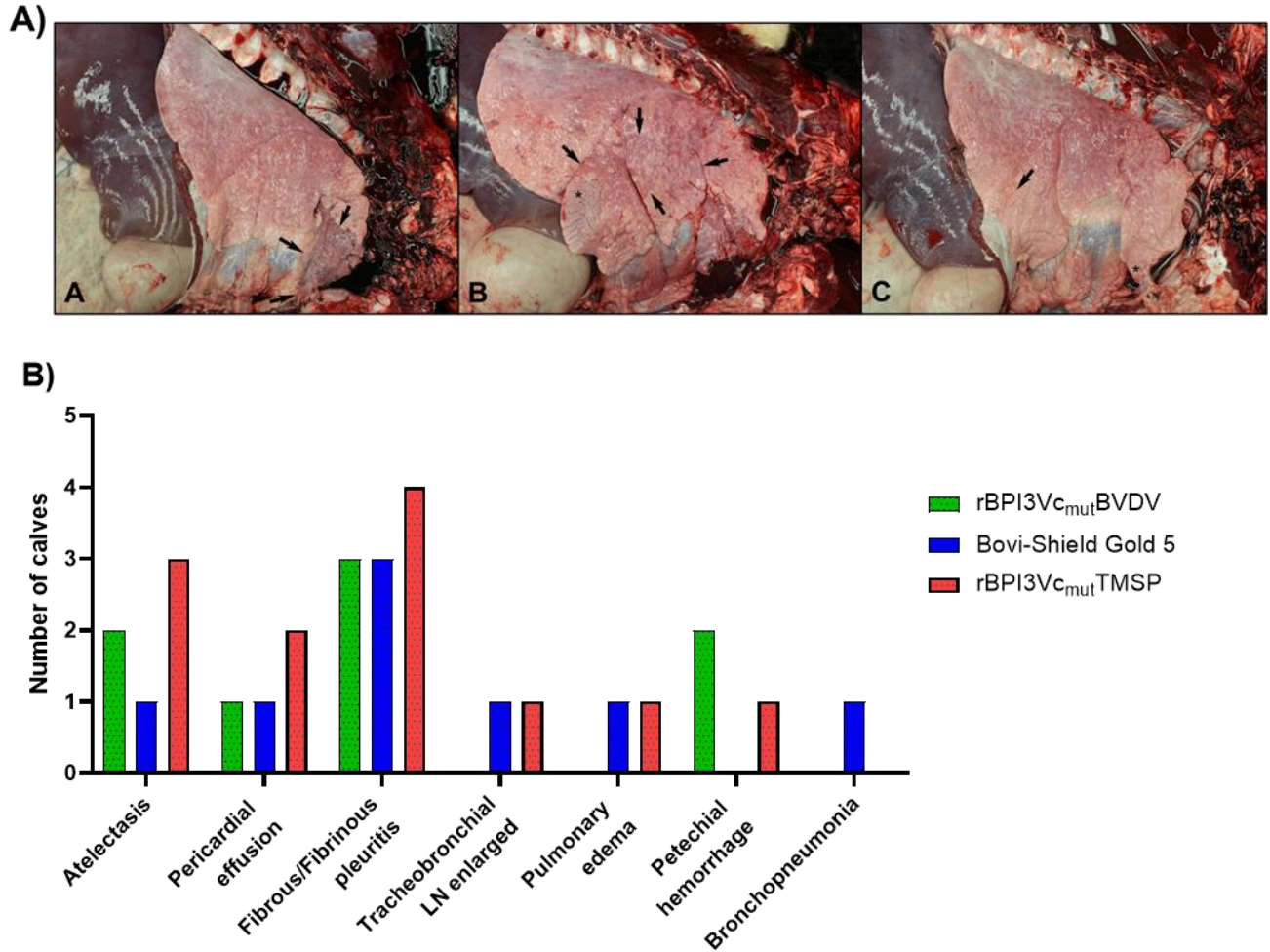


Figure 3.12. Gross lesions.

A) Gross images of lung lesions showing: Panel-A: Bronchopneumonia and areas of consolidation (arrows) and multiple fibrous adhesions (*); Panel-B: Atelectasis (arrows) and interlobular emphysema (*); and Panel-C: fibrinous pleuritis (arrows) and fibrous pleuritis (*). **B)** Number of calves presenting gross lesions recorded from the thoracic cavity and lung tissues of calves necropsied 18 days post-challenge. Bars represent the number of affected animals for each treatment group.

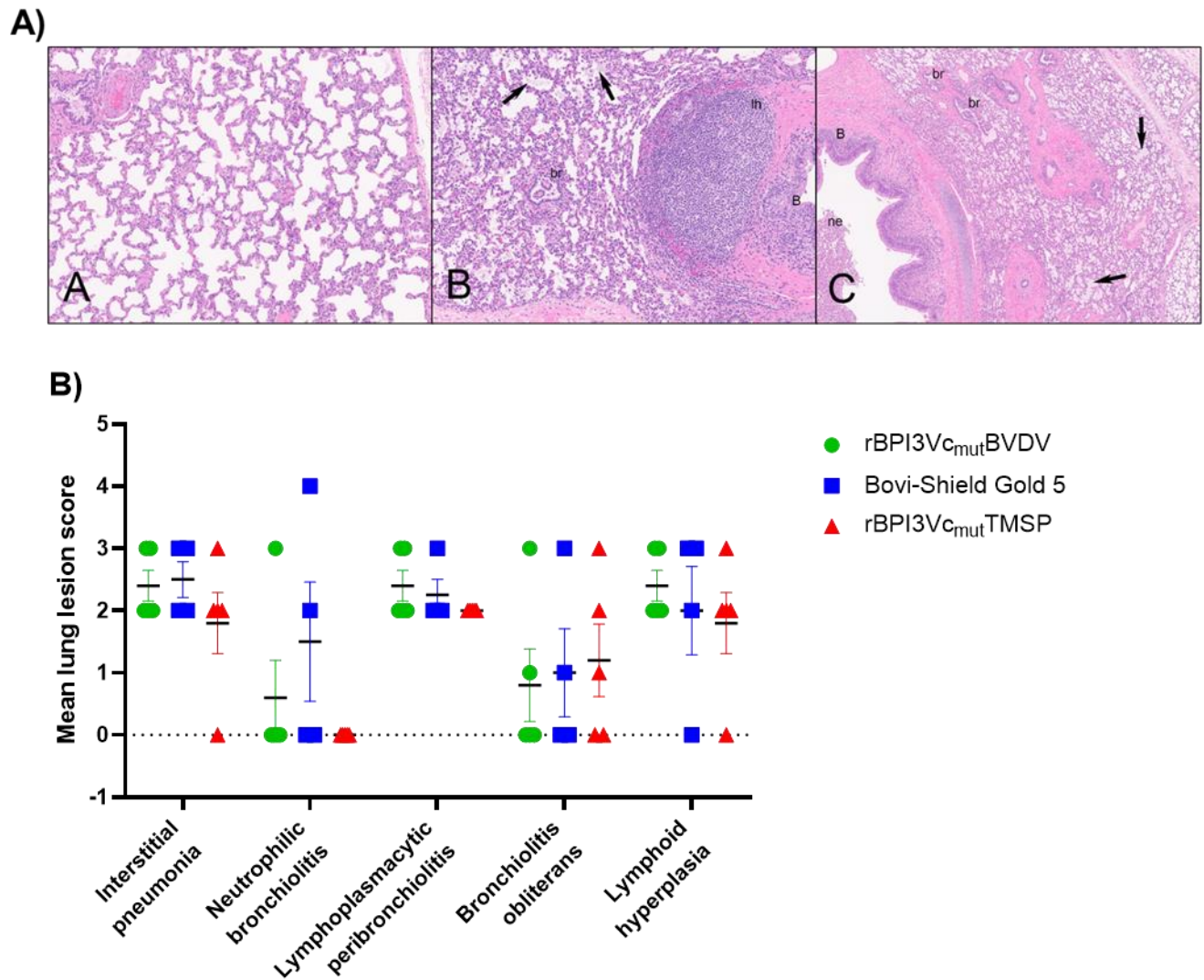


Figure 3.13. Histopathologic lung lesions.

A) Histopathologic sections showing: panel-A) diffuse mild interstitial pneumonia interstitial pneumonia, where the interstitium and alveolar septa are diffusely mildly expanded by increased infiltrating lymphocytes and plasma cells. (10x). panel-B) Diffuse mild to moderate interstitial pneumonia, chronic lymphoplasmacytic bronchitis (B) and bronchiolitis, lymphoid hyperplasia (lh) of the bronchiolar associated lymphoid tissue (BALT), pulmonary edema (arrows), and early bronchiolitis obliterans (br) (10x). Panel-C) Lesions similar to panel-B along with a neutrophilic exudate (ne) within bronchus (B, bronchitis), characteristic of Broncho-interstitial pneumonia at (4x).

B) Individual lung lesions were scored from sections of the cranial, middle, and caudal lobes of the right and left lung, based on the severity of lesions as follows: (0= normal; 1 = lesion is present;

2 = mild; 3 = moderate; 4 = severe). Individual lung lesion score for each animal is plotted. Bars represent mean lung lesion score for each group.

Chapter 4- Conclusion

Bovine respiratory diseases pose a significant threat to the dairy and beef cattle industries, causing substantial economic losses due to cattle morbidity and mortality from an early age (Lemon & McMenamy, 2021). Vaccination is a primary strategy for disease control; however, current commercial vaccines against BPI3V and BVDV show limited efficacy against diverse field strains. Despite decades of use, genetic and antigenic drift have reduced the ability of these vaccines to confer broad cross-neutralization (R. Fulton et al., 2017; R. W. Fulton, Cook, Payton, Burge, & Step, 2020; Neill, Ridpath, & Valayudhan, 2015; Neill et al., 2019).

A virus neutralization titer of 1:32 is considered protective against BPI3V (Ellis, 2010; Frank & Marshall, 1971; Marshall & Frank, 1971; Masic et al., 2020), while a titer of 1:128 is required for protection against BVDV (R. W. Fulton et al., 2020). Globally, MLVs and KVs are commonly used for BPI3V and BVDV control. In the United States, BPI3V-A-specific reference serum from vaccine strain has VN titers ($\geq 1:32$) against some homologous BPI3V-A strains (Neill et al., 2015). However, lower titers (ranging from $<1:8$ to $1:16$) were observed against certain homologous BPI3V-A strains and heterologous BPI3V-B and BPI3V-C strains, indicating gaps in vaccine coverage (R. Fulton et al., 2017; Neill et al., 2015). Furthermore, several studies around the globe have shown that genotype-specific MLVs are ineffective at conferring cross-protection against diverse BVDV strains (Alpay & Yeşilbağ, 2019; Baccili et al., 2019; Botton, Da-Silva, Brum, Weiblen, & Flores, 1998; Grange, Mindeguia, Gisbert, & Meyer, 2023). In the United States, MLVs are formulated using BVDV-1a or -2a strains (R. W. Fulton et al., 2020; Melkamsew, Tessema, & Paeshuyse, 2025), yet, when tested against emerging BVDV-2c genotypes, they induce significantly lower VN titers, which fall below the protective threshold against both BVDV-2c

genotypes and even against other homologous and heterologous genotypes (R. W. Fulton et al., 2020; Neill et al., 2019).

Although recent advancements have led to the development of improved MLVs and subunit vaccine candidates, some of which have already been tested in feedlot cattle and already approved for use (Bellido et al., 2024; Masic et al., 2020; Masic et al., 2021; Taberner et al., 2024; Tapiolas et al., 2024), their main shortcoming is that they are based on a representative single-strain and may provide minimal or no cross-protection against diverse strains, particularly due to antigenic drift. Given that antigenic drift is an ongoing challenge, next-generation vaccines should consider platforms that offer the ease and versatility of quickly refining the antigenic determinants as needed for improved broad protection against diverse strains. Given this scenario, two primary gaps were identified: 1) the lack of an effective cross-protective BPI3V vaccine and 2) the lack of an effective cross-protective BVDV vaccine.

Study 1 aimed at developing a contemporary live attenuated BPI3Vc virus vector and testing whether intranasal delivery of BPI3Vc-vectored novel mosaic F2 and HN2 antigens could safely confer broad protection against wild-type BPI3V-a, -b, -c strains. We developed a BPI3V vaccine vector based on a genotype C strain that exhibited a temperature-sensitive attenuated phenotype *in vitro*. Novel mosaic F2 and HN2 antigens were designed based on the consensus of all BPI3V-a, -b, and -c sequenced genomes as of 2019. These determinants contain protective B cell neutralizing epitopes (Collins, 1996; Kasel, Frank, Keitel, Taber, & Glezen, 1984; Toth & Frank, 1988) and IFN- γ -inducing CD4⁺ T cell epitopes that activate CD8⁺ T cells (Zhang et al., 2024; Zhang et al., 2023). We demonstrated that the vector backbone is relatively stable and maintains transgene expression over several passages, which are attractive traits for a vaccine vector (Bukreyev, Skiadopoulos, Murphy, & Collins, 2006; Phan et al., 2017). Additionally, the

vector replicon retained its infectivity and immunogenicity, eliciting neutralizing antibodies against self and transgene-encoded antigens. Our findings show that the rescued recombinant BPI3V_{cmut}F2HN2 prototype vaccine outperformed the commercial Bovilis Nasalgen PI3 vaccine in inducing and maintaining mucosal antibody responses, as well as eliciting potent neutralizing antibodies against BPI3Va-c strains, with exceptionally high VN titers, higher than even those elicited by commercial MLVs in the United States (Masic et al., 2020). Upon challenge with a reference BPI3V genotype-C strain, TVMDL16, the rBPI3V_{cmut}F2HN2 vaccine was more effective in reducing viral loads in nasal secretions and in blood of immunized calves. The outcome of this study lays a foundation for not only improved broad protection against BPI3V in future, but also other pathogens that cause BRD.

In this regard, the second study aimed at utilizing the live attenuated BPI3V_{cmut} vector to test whether intranasal delivery of rationally designed novel mosaic E2, NS2-3, and NS4-5 antigens could safely confer broad protection against diverse BVDV strains. The novel mosaic E2-NS2-5¹⁻² antigens were designed from more than 200 sequenced BVDV-1a, -1, and -2 genomes as of 2019. These determinants contain defined protective B cell neutralizing epitopes (Deregt, van Rijn, Wiens, & van den Hurk, 1998), CD4⁺ T-cell epitopes that induce strong IFN- γ secretion (Collen, 2000.; Riitho et al., 2017), MHC *DR*-restricted T cell epitopes (Beer, 1997.; Collen, 2000.; Deregt et al., 2005; Hegde, 1997; Howard, 1992.), and potent IFN- γ -inducing CD8⁺ T cell epitopes that are highly conserved among diverse BVDV strains (Sangewar et al., 2021). Our findings showed that rescued rBPI3V_{cmut}E2-NS2-5¹⁻² viruses display the E2 ectodomain on the surface of infected cells, and the presence of ~ 5kb transgenes did not adversely affect viral replication, with stable expression of transgenes over several *in vitro* passages. A cocktail of the rBPI3V_{cmut}BVDV viruses outperformed the commercial Bovi-Shield Gold 5 vaccine in eliciting potent cross-

neutralizing antibodies against representative BVDV-1a, -1b, and -2 strains, with notably higher VN titers against strains of the most prevalent BVDV-1b genotype, along with all tested BVDV-2 genotypes (which constitute the recent emergent genotypes (Neill et al., 2019). Upon challenge with a prevalent BVDV-1b strain (BVDV-California), the rBPI3V_{cmut}BVDV prototype vaccine significantly reduced viral loads in blood of vaccinated calves. This data supports use of the live-attenuated BPI3Vc-vector for development of contemporary broadly protective BVDV vaccines that can easily be upgraded for better disease management and cattle productivity.

Therefore, the broader objective of this study was to develop contemporary prototype vaccines capable of safely conferring cross-protection against diverse BPI3V and BVDV strains. Ideally, such vaccines should contain shared and unique protective B-cell and T-cell epitopes to offer broader epitope coverage for protective immunity. Such a vaccine should ideally also be administered intranasally, activating local and systemic immunity even in the face of maternal antibodies, especially in young calves, which are most vulnerable to infection as maternal antibodies wane. The main advantages of the platform described in this study are: i) the versatility it offers in easily refining antigenic determinants based on future needs, to broaden coverage or to capture new targets from emerging isolates; and ii) an immunogenic backbone vector that already elicits potent neutralizing antibodies against BPI3V, making it ideal for developing other BRD related vaccines. However, further refinement is needed to enhance this platform's efficacy.

First, an optimal immunization dose, immunization regimen, and duration of immunity need to be empirically determined. A 10^7 TCID₅₀ challenge dose in colostrum-deprived calves was initially observed to cause disease, indicating its sufficiency to be recognized as foreign. Other recent studies in calves using MLVs against BPI3V have used priming doses ranging from 10^5 TCID₅₀ to $10^{8.17}$ TCID₅₀, (Masic et al., 2020; van Rooij et al., 2023; Vangeel, Ioannou, Riegler,

Salt, & Harmeyer, 2009). Given that these studies targeted protection against homologous viruses, this pilot proof-of-concept study utilized doses starting from 10^8 TCID₅₀ for the BPI3V efficacy study and 10^9 TCID₅₀ for the heterologous BVDV efficacy study. However, it is possible that these doses could have been higher than needed, and only a single boost would be enough to confer protection, highlighting the need for further investigation. Furthermore, the duration of immunity needs to be established in order to optimize re-vaccination schedules while ensuring long-term protection.

Another critical consideration is the need for a proof-of-concept study to determine the efficacy of administering both the BPI3V-specific rBPI3V_{Cmut}F2HN2 vaccine along with the BVDV-specific rBPI3V_{Cmut}BVDV vaccine as a bivalent formulation, using a single refined BVDV-protective antigen. This would help determine the optimal immunization route, whether intranasal, parenteral, or a combination of both. This may help to inform whether an initial intranasal route and a subsequent booster parental route is more efficacious, as has been earlier suggested with BPI3V MLVs (Ellis, 2010).

Lastly, developing this platform with a component capable of Differentiating Infected versus Vaccinated (DIVA) cattle may be helpful. This could be achieved by incorporating immunogenic epitopes into the vaccine design and developing corresponding ELISA or Lateral Flow diagnostic tests to detect these vaccine-specific antibodies in immunized calves. One such defined epitope is the QPQPQPEPQPEP, obtained from the polymorphic immunodominant molecule of *Theileria parva*. This epitope is recognized by cattle infected with *T. parva*, but the parasite is absent in the United States, making it a suitable marker for DIVA-compatible vaccine (P. Toye, Nyanjui, Goddeeris, & Musoke, 1996; P. G. Toye et al., 1995).

Taken together, the advancements taken in improving broadly protective BPI3V and BVDV vaccines contribute to better disease management and ultimately enhance productivity.

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