

**GENERATION OF RECOMBINANT CAPRIPOXVIRUS VACCINES: THE
DEVELOPMENT OF A BIVALENT PESTE DES PETITS RUMINANTS VACCINE
AND A DIFFERENTIATING INFECTED FROM VACCINATED ANIMAL VACCINE**

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ABSTRACT

Capripoxvirus (CAPV) represents a genus of the *poxviridae* family which includes: sheep pox virus (SPPV), goat pox virus (GTPV), and lumpy skin disease virus (LSDV) affecting sheep, goats, and cattle respectively. Peste des petits ruminants (PPR) is a viral disease of ruminants caused by the morbillivirus, peste des petits ruminants virus (PPRV). These World Organization of Animal Health notifiable diseases continue to infect animals causing morbidity and mortality leading to direct and indirect economic losses in endemic countries. Currently, vaccination using live attenuated vaccines is used to control these diseases.

Due to the concurrent prevalence of CAPV and PPR, the use of a bivalent vaccine would be a cost effective approach to control the diseases in sheep and goats. A live attenuated CAPV vaccine was genetically modified to express an antigenic protein from PPR. This was done using the Romanian CAPV vaccine and inserting the hemagglutinin (H) protein of PPR (Moroccan) in the IL-10 homologue gene region. This project offers a solution for the eradication of PPR as provides a thermostable vaccine which can be used instead of live attenuated PPR vaccines which are thermos-labile. Additionally, it provides a cost effective method to control CAPV diseases and PPR.

The second project deals with differentiating infected from vaccinated animal vaccines (DIVA). Due to the complete immune response generated by immunized animals to conventional vaccines, it is not possible to use existing serological tests to identify whether an animal has been vaccinated or infected. Two live attenuated CAPV vaccines were utilized to generate these vaccines by attempting to knock out what was believed to be a non-essential gene. These attempts to knock out the CP25 gene of both LSDV and SPPV were not successful. It was determined that the CP25 protein is likely essential for virus replication. This project is important since it would provide an improved solution to respond to CAPV outbreaks in endemic and non-endemic countries. The current spread of LSDV throughout Europe and previous reported outbreaks of SPPV, GTPV, and LSDV in many countries highlights the need for a DIVA vaccine to improve the control of CAPV diseases.

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DEDICATIONS

This dissertation is dedicated to:

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LIST OF ABBREVIATIONS

BEFV – Bovine ephemeral fever virus
BTV – Bluetongue virus
CAPV – Capripoxvirus
CMC – Carboxymethyl cellulose
CPE – Cytopathic effect
CRISPR – Clustered regularly interspaced short palindromic repeats
DIVA – Differentiating infected from vaccinated animals
DMEM – Dulbecco's modified eagle medium
DMSO - Dimethyl sulfoxide
DNA – Deoxyribonuclease
DNTP – Deoxynucleoside triphosphate
DPV – Day(s) post vaccination
DTT – Dithiothreitol
E. coli – *Escherichia coli*
EDTA – Ethylenediaminetetraacetic acid
EEV – Extracellular enveloped virus
eIF2 α – Eukaryotic transition initiation factor 2 alpha
ELISA – Enzyme linked immunosorbent assay
F – Fusion
FBS – Fetal bovine serum
FMD – Foot and mouth disease
GFP – Green fluorescent protein
GCES – Global Control and Eradication Strategy
Gpt – Guanosine phosphoribosyl transferase
GTPV – Goat pox virus
H – Hemagglutinin
HN – Hemagglutinin-neuraminidase
HSV – Herpes simplex virus
IFN- γ – Interferon gamma

IL-4 – Interleukin 4
IMV – Intracellular mature virus
Kb – Kilobase
Kbp – Kilobase pair
LacZ – β -galactosidase gene
LAMP – Loop-mediated isothermal amplification LSDV – Lumpy skin disease virus
M – Molar
M Protein – Matrix protein
MOI – Multiplicity of infection
MOPS - 3-Morpholinepropanesulfonic acid
N – Nucleoprotein
NCBI – National Center for Biotechnology Information
ng – Nanogram
nm – Nanometer
OA3.Ts – Ovine testes cell line
OIE – World Organization of Animal Health
PBS – Phosphate buffered saline
PCI – Phenol:chloroform:isoamyl
PKR – Protein kinase R
PPR – Peste des petits ruminants
PPRV – Peste des petits ruminants virus
PVDF – Polyvinylidene difluoride
PVRL4 – Polio receptor-like 4
RNA – Ribonucleic acid
RPM – revolutions per minute
RPV – Rinderpest virus
RT – Room temperature
RT-PCR – Reverse transcriptase polymerase chain reaction
RVFV – Rift Valley fever virus
SiRNA – Small interfering ribonucleic acid
SPPV – Sheep pox virus

SRMV – Small ruminant morbillivirus

TBE – Tris-borate-EDTA

TCID – Tissue culture infectious dose

TE – Tris EDTA

TK – Thymidine kinase

V - Volt

VACV – Vaccinia virus

VLP – Virus like particle

VNT – Virus neutralization test

μL – Microliter

1. INTRODUCTION

Chapter 1: Capripoxviruses

1.1.1 Poxviruses

Poxviruses are large DNA viruses belonging to the *poxviridae* family. They are divided into two subfamilies: *entomopoxvirinae* which infect invertebrates and *chordopoxvirinae* which infect vertebrates (Joklik, 1966) (Moss, 1974) (Buller & Palumbo, 1991). The classification of poxviruses first began with the grouping of all viruses characterized by pocks or lesions on the skin. However, due to the presence of other viruses which presented with similar phenotypes such as chickenpox and syphilis, more stringent criterion were applied in their classification resulting in the widely known taxonomy used to date (Buller & Palumbo, 1991).

Chordopoxvirinae are relatively large, brick shaped, and consist of viruses with genomes ranging from 130kb – 300 kb (McFadden, 2005). *Chordopoxvirinae* are further divided into eight genera, with each genus having varying degrees of host specificity (McFadden, 2005). Poxviruses are unique from other DNA viruses in their exclusive use of the cytoplasm to replicate (Joklik, 1966) (Carroll & Moss, 1997). The use of the cytoplasm to replicate has contributed to the sophisticated nature of poxviruses due to the expectation of the viruses to encode all essential functions without utilizing host genetic machinery which is found in the nucleus (Traktman, 1990). Nonetheless, they are also able to utilize the host cell to maximize the efficiency of cytoplasmic DNA replication (Schramm & Locker, 2005). Poxviruses are also unique in that they encode numerous proteins which directly interact with host processes at the cellular and systemic level (Buller & Palumbo, 1991). Vaccinia virus (VACV), the prototype of the *poxviridae* family and vaccine against smallpox, is often solely used to elucidate information regarding DNA replication, infection, virus lifecycle, and virus tropism of chordopoxviruses (Schramm & Locker, 2005) (Moss, 2013).

Poxviruses, specifically chordopoxviruses, are promiscuous in terms of cell entry as there are no specific host cell receptors used by the viruses to mediate cell entry (McFadden, 2005). Contrary to common knowledge about other families of viruses, their specificity lies in which host cells are capable of supporting the replication of poxviruses. Essentially, poxvirus tropism is

determined downstream of virus entry into the host cell (McFadden, 2005). While the mechanism of entry is not well understood, the binding of the virion (viral infectious particle) is determined by a number of virion proteins or glycosaminoglycans on the host cell surface and/or extracellular matrix non-specifically (Smith, Vanderplasschen, & Law, 2002) (Schramm & Locker, 2005). There are two distinct virions that can infect the host cell, the intracellular mature virus (IMV), and the extracellular enveloped virus (EEV). The two virions differ in their constituent surface glycoproteins and wrapping membranes (Vanderplasschen, Hollinshead, & Smith, 1998) (Locker, et al., 2000). The IMV consists of the viral DNA genome encapsulated in a proteinaceous core consisting of 60 proteins; this is encased by an outer lipoprotein membrane consisting of 25 proteins (Laliberte & Moss, 2010). While transiting through the cytoplasm, the IMV acquires two membrane bilayers to result in the EEV, a less abundant enveloped virion - one of these membranes is removed during exocytosis (Laliberte & Moss, 2010). IMV and EEVs are antigenically dissimilar due to the presence of an additional membrane with over six unique proteins on the EEV (Smith, Vanderplasschen, & Law, 2002) (Laliberte & Moss, 2010). EEVs play an important role in efficient virus dissemination and protection against host immunity whereas IMVs are believed to be important in transmission between hosts (Smith, Vanderplasschen, & Law, 2002). Upon infection or entry of host cell, both IMV and EEV release their core containing the viral genome into the cytoplasm. mRNAs are then transcribed within the core and released into the cytoplasm for translation and un-coating of the core occurs upon synthesis of viral proteins (Schramm & Locker, 2005).

Poxvirus DNA synthesis occurs within two hours of infection in specific sites in the cytoplasm referred to as 'factories' (DeLange & McFadden, 1990) (Katsafanas & Moss, 2007). Factories are the site of virion assembly, transcription, and translation of viral mRNAs; the number of factories directly corresponds to the multiplicity of infection (MOI) at which permissive cells are infected (De Silva & Moss, 2005) (Katsafanas & Moss, 2007). The lifecycle of poxvirus consists of early gene expression, DNA replication, and late gene expression (Traktman, 1990). While early transcription proteins are packaged in virions, DNA replication proteins are translated from early mRNAs in these factories (Yang, et al., 2011). DNA replication then occurs with progeny DNA serving as template for intermediate and late stage genes (Yang, et al., 2011) (Moss, 2013). Fully permissive replication of poxvirus consists of three stages of viral mRNA and protein

synthesis: early, intermediate, and late; these stages are then followed by the assembly of infectious particles (McFadden, 2005).

1.1.2 Epidemiology

Capripoxviruses (CAPVs) are a genus of the *poxviridae* family under the *chordopoxvirinae* subfamily. There are three animal virus species that are included in CAPVs: sheep pox virus (SPPV) which mainly affects sheep, goat pox virus (GTPV) which mainly affects goats, and lumpy skin disease virus (LSDV) which affects cattle (Kitching, Bhat, & Black, 1989) (Kitching R. , 2003) (Babiuk S. , Bowden, Boyle, Wallace, & Kitching, 2008). Capripoxviruses are large double stranded DNA viruses much like other viruses found in the *poxviridae* family (Lefkowitz, Wang, & Upton, 2006). SPPV and GTPV have genomes spanning approximately 150 kilobase pairs (kbp), whereas, the LSDV genome spans 151kbp. SPPV, GTPV, and LSDV share over 96% nucleotide identity, however, LSDV contains nine functional genes that are disrupted in SPPV and GTPV (Tulman, et al., 2001) (Tulman, et al., 2002). The loss of gene functionality in SPPV and GTPV has led to the hypothesis that CAPVs originated from an LSDV-like ancestral virus that was present long before the current LSDV was first described (Biswas, et al., 2019).

SPPV was initially described during the second century in central Asia after which it spread to nearby countries and continued until it reached Europe (Bhanuprakash, Indrani, Hosamani, & Sign, 2006) (Ylmaz, Arayici, Maharramov, & Mustafaeva, 2016). SPPV and GTPV are known to be endemic in a significant portion of the globe including north and central Africa, India, southwest and central Asia (Tuppurainen, et al., 2014). Despite the confinement of SPPV and GTPV in endemic regions, outbreaks of the diseases in regions bordering endemic countries have been previously reported (Beard, et al., 2010) (Karapinar, Ilhan, Dincer, & Yildirim, 2016). In contrast, LSDV was first described much later than SPPV and GTPV, in 1929 in sub-Saharan Africa (Woods, 1988). It was believed to be a disease affecting Africa and was confined to all of Africa excluding some north African countries such as Morocco, Algeria, Tunisia, and Libya for a number of years (Biswas, et al., 2019). LSDV has now spread out of Africa into neighboring Middle East countries and has continued to spread to Eastern Europe and Asia (Tuppurainen, et al., 2014) (Mercier, et al., 2018). LSDV has now been reported in numerous countries including Greece, Bulgaria, Serbia, Kosovo, Albania, Armenia, Montenegro, the Caucasus Region,

Kazakhstan, Azerbaijan, Vietnam, China, and most recently has been reported in India, Bangladesh and Nepal (Mercier, et al., 2018) (EFSA, et al., 2019) (Teffera & Babiuk, 2019). The accelerated spread of LSDV into countries previously considered non-endemic is quite worrisome because of the implications further spread of LSDV will have into bordering nations (Madhavan, Venkatesan, & Kumar, 2016). It has also been reported that European breeds of ruminants and cattle may be more susceptible to CAPVs (Bhanuprakash, Indrani, Hosamani, & Sign, 2006). Specifically, high-producing dairy cattle and European breeds of sheep and goats have a higher susceptibility to CAPV infection (Tuppurainen E. , et al., 2015). Additionally, CAPVs are attributed to being one of the limiting factors in the genetic improvement of cattle and small ruminants and subsequently the absence of large scale production units in endemic areas (Tuppurainen E. , et al., 2015). The effects of CAPVs coupled with the rapid spread of LSDV could only be mitigated through effective vaccination campaigns as LSDV will inevitably spread into neighboring countries owing to a varying number of factors (Mercier, et al., 2018).

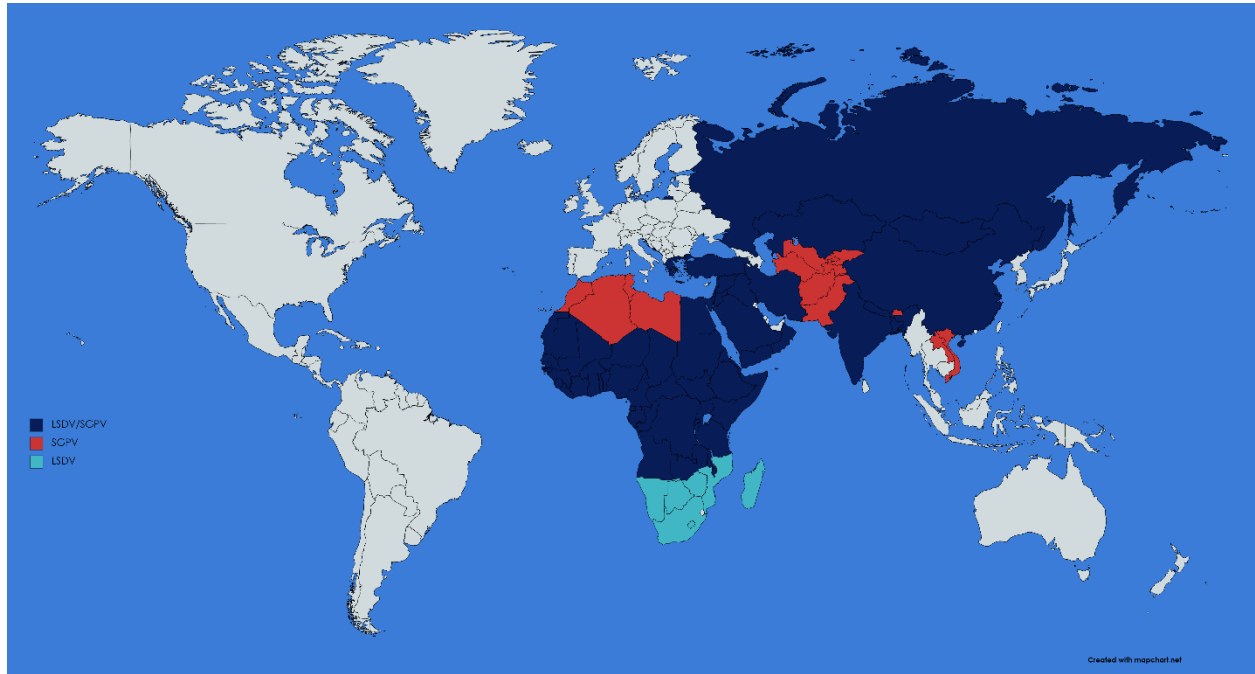


Figure 1.1 Current Prevalence of LSDV, SPPV, and GTPV globally. The diseases are concentrated in Africa, Asia, the Middle East, and parts of Europe. Map was creating using MapChart.net®

1.1.3 Transmission

The transmission of SPPV and GTPV can occur via aerosol, contact with contaminated material such as bedding, and/or direct contact between infected animals (Kitching P. , 1983) (Babiuk S. , Bowden, Boyle, Wallace, & Kitching, 2008). The stable nature of the viruses may also result in the persistence of SPPV and GTPV for a longer period of time allowing for the infection of naïve small ruminants (Tuppurainen E. , et al., 2015). LSDV is believed to be transmitted through vectors including ticks and flies but may also be transmitted by direct contact between animals (Babiuk S. , Bowden, Boyle, Wallace, & Kitching, 2008) (Chihota, Rennie, Kitching, & Mellor, 2001) (Mercier, et al., 2018) (Hota, et al., 2018) (Babiuk S. , et al., 2009). The spread of LSDV via vectors has been shown experimentally by Chihota *et al* and others; it has also been shown to be able to survive in mosquitoes without losing its titre for a period of six months (Tuppurainen & Oura, 2011), (Chihota, Rennie, Kitching, & Mellor, 2001). This is further supported by the presence of high titer of virus on the skin of infected animals (Bowden, Babiuk, Parkyn, Copps, & Boyle, 2008). The most likely vectors involved in the transmission of LSDV are stable flies, mosquitos and hard ticks (Sprygin, Pestova, Wallace, Tupppurainen, & Kononov, 2019). Because CAPVs have a tissue tropism for epithelial tissue, it allows transmission of the virus by insect or arthropod vectors to be efficient in the absence of replication in the vector (Bowden, Babiuk, Parkyn, Copps, & Boyle, 2008). Though mechanical transfer might not play as significant of a role, spread of SPPV and GTPV is aided by the ability of the respective viruses to remain in wool for up to two months and on the premise of areas where the animals reside for an even longer period of time (Kitching P. , 1983). The exponential spread of LSDV from the Middle East is suspected to be caused by increased migration partially due to conflicts; this leads to the movement of refugees and accompanying farm animals supplemented by the dispersion of insect vectors (Mercier, et al., 2018) (Tuppurainen E. , et al., 2015).

1.1.4 Infection, immunity, and clinical symptoms

The severity of disease caused by CAPVs has resulted in the classification of these diseases as World Organization for Animal health (OIE) notifiable. They are also considered potential agro-terrorism/animal bioterrorism agents (Madhavan, Venkatesan, & Kumar, 2016) (Bhanuprakash, Indrani, Hosamani, & Sign, 2006) (Tuppurainen & Oura, 2011). Animals affected by CAPVs

have similar clinical symptoms which include hyperthermia (fever) and the development of pox lesions on their skin and internal organs. These symptoms start after an average incubation of 6-7 days (Babiuk S. , Bowden, Boyle, Wallace, & Kitching, 2008) (Tulman, et al., 2002) (Tulman, et al., 2001) (Babiuk S. , et al., 2008b). The pox lesions can also become vesicles in some cases (Hamidouche, et al., 2018). Cells infected with CAPV have distinct morphology which includes the presence vacuolated cytoplasm and nuclei with the possible presence of multiple cytoplasmic inclusion bodies (Embury-Hyatt, et al., 2012). The specific route of cell entry is not studied in CAPVs, however, extrapolating from orthopoxvirus members, a genus in the *chordopoxvirinae* subfamily, it is most likely endocytic (Moss, 2012). CAPV antigen has been observed not only in epithelial cells within the skin and mucosal organs but in a wide array of cell types including: fibroblasts, muscle cells, pericytes, and immune cells such as macrophages (Embury-Hyatt, et al., 2012) (Babiuk S. , et al., 2008b). The early infection of monocytes and macrophages is suspected to assist the systemic dissemination of CAPVs (Tuppurainen E. , et al., 2015) (Embury-Hyatt, et al., 2012). Secondary lesions have been shown to occur predominantly in organs such as the skin, lung, lymph nodes and the gastrointestinal tract in natural and experimental infections with the earliest lesions appearing at day 6 post infection (Embury-Hyatt, et al., 2012). The clinical symptoms not only affect the well-being and viability of the infected animal for trade and consumption but in most cases it also affects their hides permanently (Babiuk S. , Bowden, Boyle, Wallace, & Kitching, 2008) (Bhanuprakash, Indrani, Hosamani, & Sign, 2006) (Tuppurainen, Venter, & Coetzer, 2005). LSDV has an additional effect on lactation causing major losses in milk production. Temporary and permanent infertility have also been reported to occur in cattle infected with LSDV (Tuppurainen & Oura, 2011) (Tuppurainen, et al., 2014).

Little is known regarding the CAPV specific immune response triggered by host cells. However, it has been shown that CAPV infection triggers protein kinase R (PKR) and eukaryotic translation initiation factor 2 alpha (eIF2 α). This response directly correlates with lowered replication of CAPVs, specifically SPPV and GTPV (Zhao, et al., 2018). Immune response to vaccination has also been observed in the field using GTPV and SPPV vaccines against LSDV in calves (Varshovi, Norian, Azadmehr, & Ahangaran, 2017). *In vitro* examination of the cellular immune response in cattle showed the increase of lymphocyte proliferation and increase in pro-

inflammatory cytokines such as interferon gamma (IFN- γ) and interleukin-4 (IL-4) beginning 7 days after infection lasting until 21 days after infection (Varshovi, Norian, Azadmehr, & Ahangaran, 2017). Long-term immunity against a CAPV infection is believed to be mainly cell-mediated, but humoral immunity is also known to play a role (Kitching, Hammond, & Taylor, 1987) (Tuppurainen E. , et al., 2015). The important protective role of antibodies against CAPV has been demonstrated via a passive transfer of sera to naïve sheep from SPPV and/or GTPV infected sheep where recipient sheep were fully protected against a virulent challenge of GTPV (Kitching R. , 1986). Furthermore, a vertical transfer of protection was observed against a virulent challenge with GTPV in lambs born from sheep that had been previously infected with different isolates of GTPV and SPPV (Kitching R. , 1986). However, in the case of LSDV it has been shown in calves from vaccinated cows that after 2-3 months, maternal antibodies against LSDV wane (Agianniotaki, et al., 2018).

SPPV and GTPV are associated with a relatively high morbidity and mortality (Madhavan, Venkatesan, & Kumar, 2016). LSDV is usually associated with a high morbidity and low mortality - generally less than 10%, although morbidity rates of up to 40% have been previously reported (Tuppurainen & Oura, 2011) (Babiuk S. , et al., 2008b). Mortality and morbidity rates while generalized are still dependent on factors such as the particular virus, host susceptibility, and the environment (Hota, et al., 2018) (Smith & Girish, 2002) (Madhavan, Venkatesan, & Kumar, 2016) (Kitching, Bhat, & Black, 1989). A non-immunity associated natural resistance to LSDV infection has also been reported to occur in cattle (Tuppurainen E. , et al., 2015). The damage and loss caused by CAPVs on small ruminants and cattle causes substantial economic loss due to severe production loss, trade restrictions, limitations on movement of animals, and co-ordination and implementation of vaccination campaigns (Tuppurainen & Oura, 2011) (Tuppurainen E. , et al., 2015). This not only affects countries which rely on export of small ruminants, cattle and by-products but it also impacts small scale farmers and pastoral societies whose livelihood is directly affected by the survival of their herds (Bhanuprakash, Indrani, Hosamani, & Sign, 2006) (Tuppurainen & Oura, 2011).

1.1.5 Cross-protection

While CAPVs are host specific, infections can occur between GTPV and SPPV (Tulman, et al., 2002) (Madhavan, Venkatesan, & Kumar, 2016) (Bhanuprakash, Indrani, Hosamani, & Sign, 2006), (Kitching, Bhat, & Black, 1989) (Kitching R. , 2003). In some viruses, however, the virulence observed is not uniform between the infected animal species. For example, Babiuk *et al* (2009) assessed the pathogenicity of two isolates (Yemen and Vietnam viruses) and it was shown that mortality and morbidity rates due to infection differed between sheep and goats demonstrating that variability in virulence between CAPV viruses. This is part of the reason why live attenuated vaccines must be evaluated to make sure protection is observed in all animal species that the vaccine is used in. The similarity in clinical symptoms and the cross infective nature of SPPV and GTPV could be explained by the fact that the viruses share a large degree of genomic similarity (Tulman, et al., 2002). The search of new vaccines need not be specific to each disease due to cross-protection. While it is not always the case, cross-protection is a phenomenon where immunity is conferred in more than one species through the vaccination of animals with a virus that predominantly affects a different species; in this instance goats that are vaccinated with a SPPV vaccine are protected against infections with GTPV, allowing for a more broad vaccination strategy (Kitching P. , 1983) (Tuppurainen, et al., 2014). It is also observed that CAPVs generally elicit similar antibody responses as they are antigenically similar; infection with one virus often results in immunity against the other viruses. Theoretically, the use of a single vaccine to immunize against all three viruses in the species is possible (Tuppurainen & Oura, 2011) (Kitching R. , 2003) (Babiuk S. , et al., 2008b) (Tizard, 2020), although not practiced. For LSDV the only vaccines which have demonstrated efficacy in the field are live attenuated South African Neethling LSDV vaccines (Milovanovic, et al., 2019).

1.1.6 Diagnostics

1.1.6.1 Virus Isolation

CAPV diagnosis occurs through the monitoring of animals for clinical signs and/or symptoms by the animal care takers or farmers. This is followed assessment of visual symptoms by a field veterinarian and by laboratory confirmation. CAPV infections can present as respiratory

infections with symptoms resembling pneumonia and must be differentiated from possible bacterial, parasitic and or other viral causes (Settypalli, et al., 2016). Samples are then collected from suspected animals to be tested; CAPV diagnosis is confirmed by laboratory personnel using standard virology/serological methods, with the gold standard being a virus neutralization test (VNT) (OIE,a, 2019) (OIE,b, 2019). Virus isolation is still recommended as a diagnostic test by the OIE and has been historically used as a confirmatory test to prove the presence of virus prior to the development of high throughput testing. Virus isolation involves the collection of animal samples which are then grown on permissive cells for 7-14 days to observe cytopathic effects (CPE) (OIE,a, 2019) (OIE,b, 2019). This test is not only laborious but also time consuming as it takes up to two weeks to get results. While virus isolation is recommended by the OIE as one of the standard tests to diagnose CAPVs, it is not ideal. In some non-endemic countries where use of live CAPV is prohibited, virus isolation is simply not possible (Heine, Stevens, Foord, & Boyle, 1999).

1.1.6.2 Serology

The VNT is considered a reference to not only assess the presence of disease but to also monitor immune status in vaccinated animals due to its basis in determining neutralizing immunity (Hamidouche, et al., 2018). VNTs, while robust and reliable, do not have the ability to differentiate between the three CAPVs nor differentiate vaccinated and uninfected versus infected animals (Hamidouche, et al., 2018). Several attempts have been made to develop an enzyme linked immunosorbent assays (ELISAs) able to detect anti-CAPV antibodies. An ELISA has been developed using the P32 protein of CAPV (Heine, Stevens, Foord, & Boyle, 1999) although not validated due to difficulty in the stability of the protein. As well, an indirect ELISA has been developed using whole inactivated CAPV antigen, however, this assay is not useful as the production of the antigen is too costly (Babiuk, et al., 2009). Additionally, an indirect ELISA was developed using two CAPV antigens (095 and 105) produced *in vitro* using an *Escherichia coli* (*E. coli*) expression system which exhibited very high sensitivity and specificity of antibody detection in experimentally infected animals but did not perform well with vaccinated animals (Bowden, et al., 2009). A commercial double antigen ELISA which likely uses the same two antigens has been used to characterize antibody responses to repeated lumpy skin disease vaccination (Milovanovic, et al., 2019) and bulk milk samples (Milovanovic, et al., 2020). The

use of a serological assay to differentiate between CAPVs has not been shown and remains unlikely due to the similarity between the three viruses.

1.1.6.3 Molecular Tests

There are a number of molecular tests described and used to routinely diagnose CAPVs in field and laboratory settings (Bowden, Babiuk, Parkyn, Copps, & Boyle, 2008) (Das, Deng, Babiuk, & McIntosh, 2017). Serological testing methods are usually supplemented with or replaced by molecular diagnostics often with multiplex capability (Settypalli, et al., 2016) (Das, Deng, Babiuk, & McIntosh, 2017). As well, there are novel molecular tests being studied, most of which aim to differentiate viral detection into a specific species of CAPV. A conventional polymerase chain reaction (PCR) has been developed to differentiate between SPPV and GTPV which takes advantage of a 21-nucleotide mutation in SPPV to allow for rapid differentiation of the two viruses (Lamien, et al., 2011). Although, conventional PCR tests are still in use, quantitative PCR (qPCR) has been shown to be more efficient with higher specificity (Balamurugan, et al., 2009). A high-throughput PCR protocol has also been published using a robotic DNA extraction protocol to increase sensitivity (Stubbs, et al., 2012). A single tube, PCR-like molecular assay known as a loop-mediated isothermal amplification (LAMP) assay has also been developed to detect CAPVs. The LAMP assay which is based on detection of a color change using primers that target a CAPV gene (poly(A) polymerase small subunit) showed similar diagnostic specificity to a qPCR assay (Stubbs, et al., 2012). The use of real-time PCR (RT-PCR) has exponentially increased the efficiency at which CAPVs can be detected (Haegeman, et al., 2013). Haegeman *et al* have developed three RT-PCRs that can be run in parallel, together, or separately to detect the SPPV, GTPV, and LSDV (2013). As well, mobile RT-PCR system using lyophilized reagents has been described to be able to detect CAPV DNA both in laboratory and field settings using samples from experimental and field samples positive for all three CAPVs (Armson, et al., 2015). Lastly, a high resolution melt-based assay has been designed to differentiate SPPV vaccines from field isolates and further identify CAPVs into either SPPV, GTPV, or LSDV (Chibssa, et al., 2019). There are a number of RT-PCR assays which have been developed to differentiate between vaccine and field virus (Agianniotaki, et al., 2017) (Pestova, Byadovskaya, Kononov, & Sprygin, 2018). Unfortunately these assays will not correctly identify field isolates if recombination events between vaccine and virulent strains

occur, a phenomenon which has recently been reported to occur (Sprygin, et al., 2020) (Kononova, et al., 2020).

1.1.7 Vaccines

1.1.7.1 Conventional vaccines

The most economical method to control SPPV, GTPV, and LSDV in endemic countries is through vaccination (Babiuk S. , Bowden, Boyle, Wallace, & Kitching, 2008) (Babiuk S. , et al., 2008b). Fundamentally, through the implementation of active and persistent vaccination campaigns, the goal is to increase herd immunity leading to eradication of CAPVs (Ylmaz, Arayici, Maharramov, & Mustafaeva, 2016). Limitations in the use of live attenuated vaccines in non-endemic areas as well as other factors including reactivity and side effects of vaccines opens the door for research for better CAPV vaccines (Tulman, et al., 2002). In disease-free countries stamping out (the slaughter of diseased animals) is a common practice and fairly effective method of control for SPPV and GTPV (Tuppurainen, et al., 2014); however, stamping out is not effective without vaccination for LSDV. The OIE requirement aside from the slaughter of animals includes a minimum 20 kilometer (km) surveillance zone surrounding sites where affected animals resided for 4 weeks (OIE, 2019). Nonetheless, this practice is not financially viable or efficient as a strategy in endemic countries (Tuppurainen E. , et al., 2015).

The most common and widely used method of vaccination against CAPVs is through the use of attenuated live field viruses (Spickler & Roth, 2003) (Tuppurainen & Oura, 2011). To date, the most commonly used live attenuated vaccines were developed through serially passaging in tissue culture until attenuation was achieved (Kitching P. , 1983) (Precausta, Kato, & Vellut, 1979). Live-attenuated vaccines against CAPVs are reported to provide protection for up to two years in endemic areas (Kitching R. , 2003). An example of a commonly used vaccine is one developed in 1997 by Precausta *et al* which is a Romanian isolate of SPPV that was passaged in lamb kidney cells 30 times until attenuation was achieved. This live attenuated vaccine was found to elicit high neutralizing antibody titres (Kitching P. , 1983), (Precausta, Kato, & Vellut, 1979). The vaccine is freeze-dried and does not contain an adjuvant. It can be stored for two years at 6°C allowing for flexibility in storage and production (Kitching P. , 1983). Live vaccines

are generally affordable and allow for the maintenance of over 80% herd protection with annual vaccination campaigns (Tuppurainen E. , et al., 2015).

Due to regulatory issues, vaccinations against CAPV are not in use in disease-free countries. Furthermore, in some countries, SPPV/GTPV vaccines are used in cattle as an alternative to LSDV vaccines under the assumption they will also protect against LSDV without demonstrating efficacy (Tulman, et al., 2002). This is not an ideal situation because vaccines should be evaluated in experimental animal trials and field trials before they are used in the field. There are several potential reasons for vaccine failure which include: the use of a non-effective vaccine, improper needle hygiene, contamination of vaccine with live virus, inactivation of virus due to light exposure or high temperatures, and interference of maternally derived antibodies (Tuppurainen E. , et al., 2015). There have also been reported cases where complete protection was not observed against LSDV following vaccination with the RM65 strain of SPPV (Brenner, et al., 2009). In the study conducted by Brenner *et al* (2009), they observed a fairly high percentage of beef cattle with symptoms after they were re-exposed to a LSDV infection, emphasizing the need for the re-assessment of vaccines being used for non-dairy cattle. The observed lack of efficacy of the vaccination however was not solely attributed to the vaccine as other possible factors could have played a role such as physical difficulties while attempting to collect and vaccinate beef cattle. Nonetheless, it is important to assess efficiency of current vaccines in use and their long term protection capabilities (Brenner, et al., 2009). Even with the above stated problems, the current conventional vaccines are still manageable compared to the possible use of inactivated vaccines which would only provide short term and insufficient protection against CAPVs (Tuppurainen & Oura, 2011). So far, the South African Neethling LSDV vaccines have been demonstrated to be effective at eradicating LSDV in Eastern Europe (Calistri, et al., 2020).

There have also been reports of attempts to control an outbreak with vaccination of animals with live virus isolated from infected animals. This often has negative effects and highlights the need to educate farmers on the use of readily available live attenuated vaccines as opposed to possibly inadvertently exposing animals to the disease (Kitching P. , 1983). In regions of Africa where SPPV and GTPV are not present such as South Africa, only attenuated LSDV vaccines are

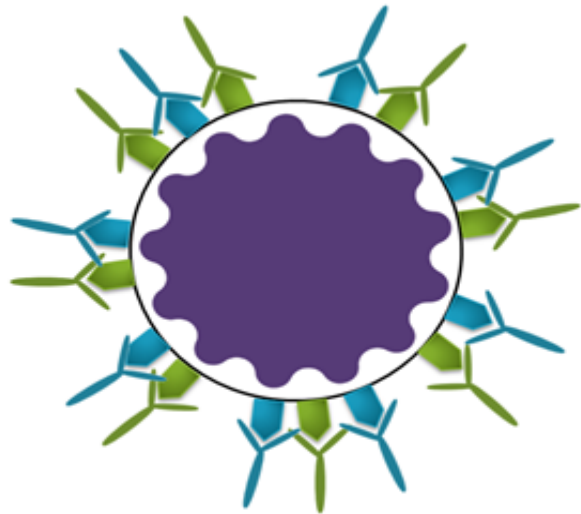
administered whereas in regions that are affected by all three viruses, both LSDV, SPPV and/or GTPV vaccines are utilized (Tuppurainen, et al., 2014). Attenuated vaccine strains currently in use include but are not limited to: Yugoslavian RM65, Romanian SPPV, and KSGP 0-180 strain. Despite the number of live attenuated CAPV vaccines in use, very few of those have been supported by animal trials in which their efficacy was experimentally determined; this may explain reports of vaccine failure or vaccine induced disease (Hunter & Wallace, 2001).

1.1.7.2 DIVA vaccines

Differentiating infected from vaccinated animal (DIVA) vaccines are an additional tool which could allow the ability to monitor the spread of CAPVs in vaccinated populations. This would enable the generation of epidemiological data which can be used to determine the effectiveness of vaccination and when vaccination can be stopped. Previously known as marker vaccines, DIVA vaccines refer to genetically altered conventional vaccines which have at least one antigenic region missing (Van Oirschot, 1999). Immunization with such vaccines results in a quantifiably different antibody response in which there is a lack of antibodies against the missing antigen, allowing for the development of a test to differentiate the antibody response (Madhavan, Venkatesan, & Kumar, 2016) (Van Oirschot, 1999) (Van Oirschot, Kaashoek, Rijsewijk, & Stegeman, 1996). This not only allows for the differentiation of vaccinated and unvaccinated animals, but it also will likely decrease the amount of wild type virus circulating in animal populations aiding in the possible eradication of a given virus (Van Oirschot, 1999) (Van Oirschot, Kaashoek, Rijsewijk, & Stegeman, 1996). Previously, the advantage of DIVA vaccines and accompanying serological tests has been experimentally shown to be effective against Aujeszky's Disease virus (Van Oirschot, Rziha, Moonen, Pol, & Van Zaane, 1986) and herpes simplex virus (HSV) (Van Oirschot, Kaashoek, Rijsewijk, & Stegeman, 1996) (Van Oirschot, Kaashoek, Maris-Veldhuis, Weerdmeester, & Rijsewijk, 1997) amongst others.



Conventional Vaccine



DIVA Vaccine

Figure 1.2 Visual representation of a conventional vaccine and a DIVA vaccine. A DIVA vaccine has one antigenic region missing therefore there will be a lack of antibodies produced to the missing protein. This allows for the development of a serological test which can be utilized to differentiate whether an animal has been vaccinated and uninfected or infected.

The idea of a DIVA vaccine requires the availability of a companion serological test. The successful development of an ELISA potentially allows for the development of a DIVA vaccine lacking a particular antigen. The development of a CAPV DIVA vaccine would be extremely useful to non-endemic countries to allow for the use of vaccination before outbreaks as it would allow countries to retain their endemic-free status (Tuppurainen E. , et al., 2015).

To date, there are no serological tools designed for CAPV specifically to differentiate between vaccinated and unvaccinated animals. However, there are molecular based methods which have been developed (Pestova, Byadovskaya, Kononov, & Sprygin, 2018) (Orlova, Shcherbakov, Diev, & Zakharov, 2006). An example is a PCR based method developed by Orlova *et al* (2006). In their study, they designed primers that were specific to SPPV endemic to Russia and the attenuated live viruses used for vaccinations. While availability of molecular diagnosis tools to differentiate between viruses is an asset, it still does not solve the problem of identifying infection in vaccinated animals. The recent demonstration of recombination between some live viruses used for vaccination and virulent field viruses has caused the molecular based DIVA methods to be unable to correctly identify vaccinated animals and uninfected specifically (Sprygin, et al., 2020).

1.1.8. Capripoxviruses as vectors

Poxviruses have a number of favorable factors that make them ideal candidates for use as recombinant vaccines (Perkus, Piccini, Lipinkas, & Paoletti, 1985) (Perkus, et al., 1991). The genetic modification of poxviruses to express proteins from other viruses has been performed as far back as 1982 when recombinant vaccinia viruses were constructed containing a thymidine kinase (TK) gene from herpes simplex virus (HSV) (Panicali & Paoletti, 1982) (Tiwari, Gautam, Bhat, & Malik, 2019). Since then poxviruses have been successfully used as vectors to vaccinate against other viruses. Namely, VACV has been used as a vector to protect wildlife against rabies (Brochier , et al., 1988) and fowl pox has been used in chickens to protect against Newcastle disease (Boursnell, et al., 1990). The factors that make CAPVs ideal vectors include their thermostability, genomic stability, large genomic size, and their efficacy as vaccines following a single immunization with a low titre of virus (Romero C. , et al., 1993) (Aspden K. , et al., 2002)

(Tuppurainen & Oura, 2011). Some of these reasons could also be attributed to the widely practiced use of live attenuated vaccines in the control of CAPVs in endemic regions. Poxviruses allow for the insertion of large non-poxvirus DNA into their genomes (Tiwari, Gautam, Bhat, & Malik, 2019). While the physiological properties of CAPVs are ideal, equally important are their tissue tropism and immunological properties. The tissue tropism of CAPVs in preferred routes of vaccination such as the epithelia and nasal turbinates allows for the possible use of less intrusive vaccination strategies (Bowden, Babiuk, Parkyn, Copps, & Boyle, 2008) (Babiuk S. , et al., 2008b). Additionally, the ability of CAPV vaccines and field isolates to elicit both humoral and cell mediated immunity following a single immunization makes them excellent vectors (Varshovi, Norian, Azadmehr, & Ahangaran, 2017) (Carroll & Moss, 1997). Although an argument can be made that other pox viruses could be used as vectors, the limited host range and non-zoonotic nature of CAPVs is advantageous over the use of other poxviruses such as VACV (Romero C. , et al., 1993) (Romero C. , et al., 1993) (Liu, et al., 2018).

The first recombinant CAPV vaccine was developed to confer dual protection against rinderpest virus (RPV) and LSDV in cattle. This vaccine was generated in lamb testicular cells using the KS-1 virus vaccine (LSDV). Permissive cells were transfected with plasmid DNA containing the fusion (F) protein of RPV and a selectable marker to replace the TK gene of LSDV; recombinant virus was isolated through rounds of plaque purification (Romero C. , et al., 1993). This vaccine was able to protect cattle completely against challenge with a virulent strain of RPV and LSDV (Romero C. , et al., 1993) (Romero C. , Barrett, Kitching, Carn, & Black, 1994). The success of the first recombinant CAPV experimental vaccine led to the development of numerous recombinant CAPV vectored vaccines against several diseases affecting small ruminants and cattle. Following the development of the first dual CAPV vaccine, recombinant KS-1 CAPV vaccine strains expressing either the F or hemagglutinin (H) genes of RPV were developed followed by their subsequent evaluation as possible dual vaccines against peste des petits ruminants (PPR) (Romero C. , Barrett, Kitching, Bostock, & Black, 1995). Both vaccines were found to be protective in experimental settings in goats against lethal challenge with PPR due to the similarity of the H and F proteins of PPR and RPV (Romero C. , Barrett, Kitching, Bostock, & Black, 1995). In 1996, it was reported that expression of the outer capsid protein VP7 of Blue tongue virus (BTV) on the KS-1 (LSDV) was able to provide partial protection of sheep against

a virulent BTV challenge (Wade-evans, et al., 1996). Recombinant CAPV generation was also done by Ngichabe *et al* (1997, 2002) where they generated LSDV expressing RPV H and F proteins followed by immunization. They reported full protection against challenge with both diseases; protection was also observed several years after initial vaccination in some animals (Ngichabe, et al., 2002). An attenuated LSDV vaccine strain (Neethling) was similarly utilized to successfully express a rabies virus glycoprotein in cattle where there was an antibody response from the cattle upon inoculation with the recombinant virus (Aspden K. , et al., 2002). Wallace and Viljoen (2005) generated recombinant LSDV (SA-Neethling) expressing the glycoproteins of rift valley fever virus (RVFV) and Bovine ephemeral fever virus (BEFV). These bivalent vaccines, which were constructed by inserting the foreign genes into the LSDV TK gene, conferred protective immunity against challenge with both viruses, respectively. The recombinant BEFV vaccine challenges resulted in the production of neutralizing antibodies similar to that elicited by commercial vaccines in cattle. This however, did not result in full protection in cattle while the RVFV recombinant vaccine did (Romero C. , et al., 1993). In 2006, Diallo *et al*, were also able to make a recombinant CAPVs (KS-1 strain) expressing the H protein from PPR, they reported that at their suggested dose, it was able to protect goats against virulent PPR. This was contrary to observations where a 100X lower dose expressing the F protein of PPR showed complete protective immunity (Diallo, et al., 2006). Subsequently, the F and H protein expressed in the TK region of a GTPV vaccine, AV41, were shown to result in PPR specific antibodies in sheep and goats while conferring protection against GTPV challenge in goats (Chen, et al., 2010). The use of CAPVs as recombinant vectors has continued with proteins from numerous infectious viruses being expressed to provide full or partial protection against virulent challenge (Kyriakis, 2015) (Chervyakova, et al., 2016) (Liu, Li, & Wang, 2019). Other protective recombinant CAPV vaccines have also been developed against more small ruminant diseases such as RVFV (Ayari-Fakhfakh, Ghram, Albina, & Cetre-Sossah, 2018).

The idea of bivalent or multivalent vaccines is very important because they allow for protective immune responses against two or more antigens of interest using a single dose of vaccine (Aspden K. , et al., 2002) (Yokoyama, Maeda, & Mikami, 1997). Due to the many advantages, CAPVs are increasingly being utilized as vectors to make recombinant vaccines (Yokoyama, Maeda, & Mikami, 1997) (Wallace, Weyer, Nel, & Viljoen, 2007). Though the exact method of

immunity elicited by the recombinant vaccines is not clearly defined, it is assumed to be both cell mediated and humoral based on previous studies on CAPVs (Ngichabe, et al., 2002). The North African KS-1 vaccine which is a LSDV and the South African Neethling LSDV vaccine have been the most commonly used CAPV vaccine strains to generate recombinant vectors (Romero C. , et al., 1993). While a number of poxviruses (canarypox, fowlpox, vaccinia virus) are used as recombinant vaccines clinically, CAPVs have yet to be used as bivalent or multivalent vectors in field immunizations (Tiwari, Gautam, Bhat, & Malik, 2019).

1.1.9 Generation of Recombinant Capripoxvirus vectors

1.1.9.1 Homologous Recombination

Homologous recombination is a commonly used method of editing genomes and has been used to successfully delete or add antigen-encoding genes into CAPVs (Carroll & Moss, 1997). Recombinant CAPV vaccine generation using homologous recombination is achieved by infection of permissive cells with a CAPV vector followed by a transfection with a transfer plasmid. The transfer plasmid contains selectable markers and the gene of interest with flanking regions for a non-essential CAPV gene, often thymidine kinase (TK) (Romero C. , et al., 1993) (Aspden K. , et al., 2002) (Wallace, Weyer, Nel, & Viljoen, 2007) (Liu, et al., 2018). Other insertion sites such as the IL-10 homologue gene (Boshra, et al., 2015), IFN- γ receptor-like gene, and a G-protein-coupled chemokine receptor subfamily homologue gene (Cêtre-Sossah , Kwiatek, & Albina, 2017), have also been used as insertion sites (Kara, et al., 2018). Furthermore, deletion of the TK as well as open reading frames 8-18 were demonstrated to further attenuate the AV41 SPPV vaccine (Zhu, et al., 2018) and deletion of the sheeppox-019 kelch like protein gene from a virulent Kazakhstan SPPV isolate was able to attenuate the virus (Balinsky, et al., 2007). These studies demonstrate that there are likely many more non-essential gene targets for use as insertion sites, which have not been demonstrated to date.

Different selection methods have previously been evaluated to determine the most appropriate markers for generating recombinant CAPVs (Wallace, Weyer, Nel, & Viljoen, 2007). The selectable markers evaluated were the *Escherichia coli* (*E. coli*) β -galactosidase gene (LacZ), use of green fluorescent protein (GFP) gene, and the use of *E. coli* xanthine

phosphoribosyltransferase (gpt) gene (Romero C. , et al., 1993) (Wallace, Weyer, Nel, & Viljoen, 2007). In terms of efficiency, it is logical to use a dual selectable marker to allow for a visual confirmation and an additional marker that allows for growth in a selective media. LacZ and GFP act as visual markers where expression of these genes demonstrates homologous recombination has occurred without any further process than infection and transfection (Wallace, Weyer, Nel, & Viljoen, 2007). Gpt is a dominant selectable marker and an added advantage as it allows for the selective growth of virus expressing the gene of interest on gpt selective media (Wallace, Weyer, Nel, & Viljoen, 2007). Selectable markers are not acceptable to use in a licensed vaccine and can be removed in one of two ways. The first method would be to insert a P11 promoter oriented in the same direction placed before and after the selectable markers (Boshra, Cao, & Babiuk, 2016). The promoter is able to drive the expression of the selectable markers while also allowing for a recombinant excision of the markers once the selective pressure is removed from the growth media of the virus during negative selection (Boshra, Cao, & Babiuk, 2016). The second method to remove selection markers is by using the cre-loxP system. Similar to the presence of the P11 promoter, it involves the incorporation of a loxP sequence on either side of the selectable markers. Then following positive selection, once a pure recombinant virus is present, it would be passaged in cells expressing cre recombinase which will recombine the two loxP sites and excise the selectable markers in between (Liu, Zhang, & Liu, 2019) (Liu, Li, & Wang, 2019).

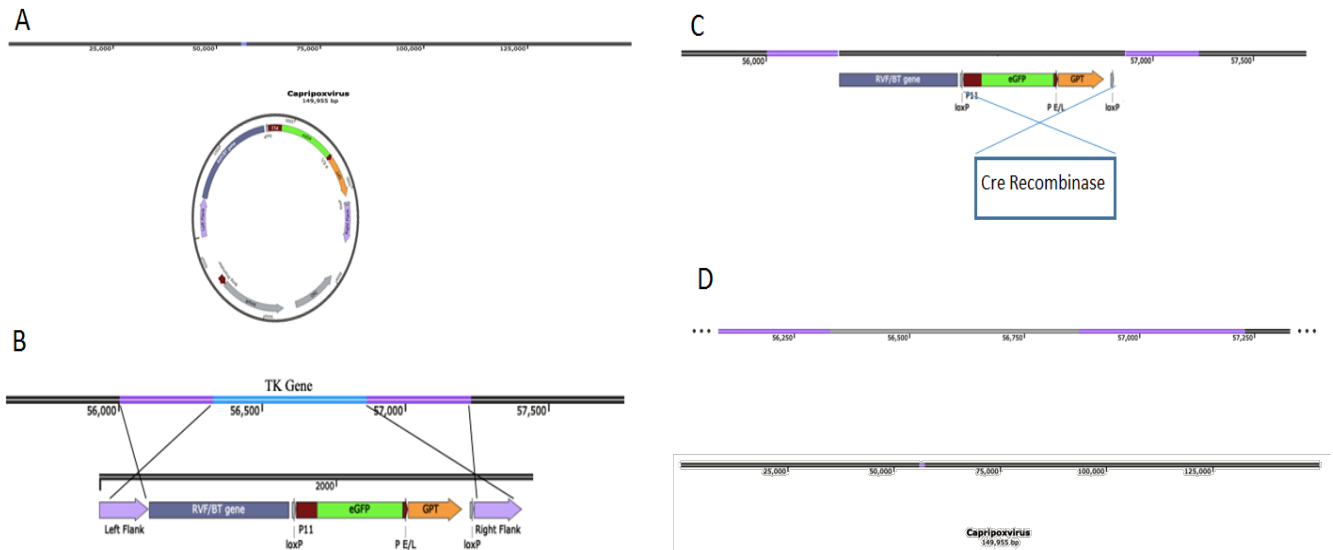


Figure 1.3 Visual representation of the generation of a CAPV expressing a foreign gene. A. The full genome of CAPV and an insertion plasmid which contains the gene of interest, two selection markers (GFP, gpt) with loxP sites on either side, and two flanking sites corresponding to genomic regions outside the CAPV gene to be replaced (eg. Thymidine Kinase). B. Alignment of the flanking regions on an insertion plasmid ideal for homologous recombination to occur with TK gene of CAPV. Homologous recombination will occur in transfected cells after which selection markers can be used to identify mutant virus. C. After rounds of positive selection, cre recombinase can be introduced using a plasmid or via cell lines expressing the protein to excise the selection markers present in the CAPV genome. D. Following successful excision of selection markers, the TK gene will have successfully been replaced with the gene of interest.

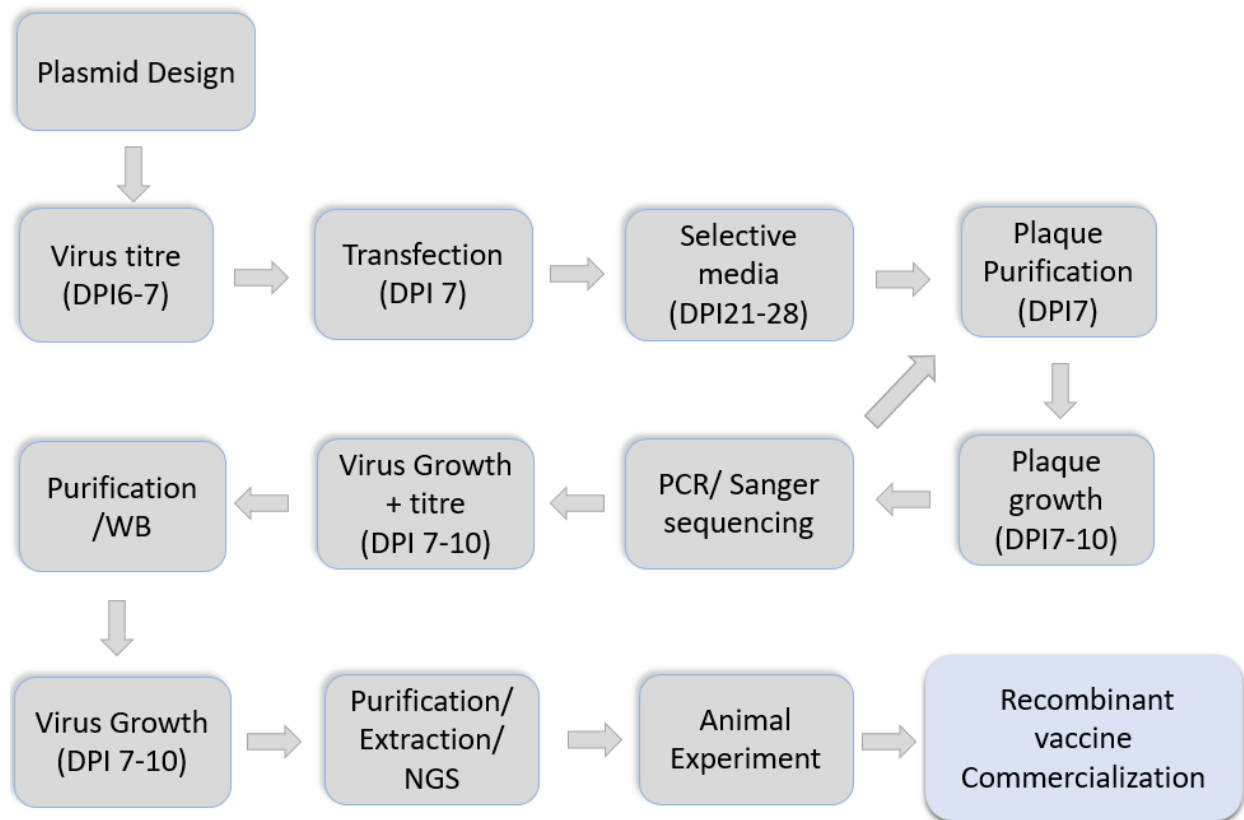


Figure 1.4 Scheme of recombinant CAPV vaccine generation. The process begins with the construction of a plasmid to either insert, replace, or remove a gene of interest. Following plasmid purification, viral plaques are grown on selective media and purified repeatedly accompanied by a molecular test to get a pure mutant plaque. Viral titers are performed at different steps of the process to measure the viral concentration to ensure proper multiplicity of infection.

1.1.9.2 CRISPR/Cas

CRISPR refers to clustered regularly interspaced short palindromic repeats. CRISPR is found in prokaryotes (bacteria and archaea) where it functions as a defense system to attack invading foreign DNA where the foreign DNA is inserted following the CRISPR sequence and CRISPR associated (Cas) genes to produce guide RNAs that then target the sequence of foreign DNA for destruction should it ever be re-introduced into the prokaryote (Yuan, et al., 2015) (Okoli, Okeke, Tryland, & Moens, 2018). CRISPR/Cas is a system that can be utilized in place of or in parallel with homologous recombination for the generation of recombinant vaccines. Although CRISPR/Cas has yet to be reported in the generation of recombinant CAPV, it has been reported in the modification of VACV (Okoli, Okeke, Tryland, & Moens, 2018) and African swine fever virus (Borca, Holinka, Berggren, & Gladue, 2018) to improve the efficiency of genetic engineering. The similarity between CAPV and VACV opens the door for the use of CRISPR/Cas system as a gene-editing tool in the process of recombinant vaccine generation (Yuan, et al., 2015) (Okoli, Okeke, Tryland, & Moens, 2018).

1.1.9.3 Synthetic generation of Capripoxviruses

A novel method of recombinant poxvirus generation has recently been demonstrated involving large scale gene synthesis (Noyce, Lederman, & Evans, 2018). The process involves the synthetic generation of large fragments of DNA up to 30kb containing overlapping sequences of at least 1kb. The fragments are synthesized in a plasmid then restricted and ligated in optimized cells with the presence of a helper virus to generate functional poxvirus (Noyce, Lederman, & Evans, 2018). Using the molecular methods stated, horsepox virus was generated from 10 fragments of synthetic DNA using Shope Fibroma virus as a helper virus (Noyce, Lederman, & Evans, 2018). The potential of this research is limitless in terms of new CAPV vaccine generation. The ability to synthetically make CAPV would allow for the modification of multiple genes at once reducing the time consuming and laborious process of plaque purification and selection. In addition, using synthetic biology will enable tailoring of the vector to enhance safety and immunogenicity.

1.2 Chapter 2. Peste des petits ruminants

1.2.1 Morbilliviruses

Morbillivirus is a genus in the *paramyxoviridae* virus family in the order *mononegavirales* (Diallo, 1990) (Banyard, et al., 2010). The virus family contains highly pathogenic viruses that affect a variety of species ranging from felines to humans (Libeau, Diallo, & Parida, 2014) (Baron, Diallo, Lancelot, & Libeau, 2016). Morbilliviruses consist of enveloped, serologically related, non-segmented, negative sense RNA viruses (Barrett & Rossiter, 1999) (Battisti, et al., 2012). They are pleomorphic in shape with envelope proteins protruding from the envelope and range from 350-600nm in size with a relatively small genome of about 16,000 nucleotides (Diallo, 1990). Morbilliviruses are extremely contagious microbes that are spread through the respiratory route and have resulted in significant loss of human and animal life throughout the world (Tatsuo, Ono, & Yanagi, 2001) (De Vries, Duprex, & De Swart, 2015). The most prominent member of the morbillivirus genus is measles virus, it has had a devastating effect on humans throughout history (Bailey, 2019). All morbilliviruses are associated with high morbidity and high mortality resulting in large outbreaks in exposed populations (De Vries, Duprex, & De Swart, 2015). It is believed that a progenitor morbillivirus originated in cattle and was passed on to humans upon the domestication of cattle resulting in the subsequent evolution of numerous morbilliviruses (Visser, Van Bresse, Barrett, & Osterhaus, 1993) (Barrett & Rossiter, 1999). The evolution and high adaptability of morbilliviruses to new hosts suggests an intrinsic capacity to constantly adapt to new hosts, however, it has been shown that the mutations required to allow for this adaption are a significant hurdle to cross any species barrier (Mateo, Navaratnarajah, Syed, & Cattaneo, 2013).

Morbilliviruses have six structural proteins and one non-structural protein. Haemagglutinin (H) and fusion (F) proteins are the structural proteins involved in host cell interaction (Diallo, 1990). The H protein is involved in the attachment of the virus to the host cell while the F protein fuses with host cell membrane to allow translocation into the host cell (Diallo, Morbillivirus group: genome organisation and proteins, 1990). One of the common traits of morbilliviruses is the use

of signaling lymphocytic activation molecule (SLAM) as a cellular receptor to enter host cells. SLAM is expressed on immune cells, specifically on immature thymocytes, memory T cells, B cells, and dendritic cells (Tatsuo, Ono, & Yanagi, 2001). The second cellular receptor used by morbilliviruses is poliovirus receptor-like 4 (PVRL4) better known as nectin-4 (Noyce, et al., 2011). However, due to the observation of infections in other cells including in the central nervous system, additional receptors may also be utilized (De Vries, Duprex, & De Swart, 2015). Morbilliviruses, although they result in incredibly high mortality, do not remain infectious after an acute infection. They result in life-long immunity once hosts recover; therefore, a continuous supply of susceptible hosts is required for their maintenance (Barrett & Rossiter, 1999).

1.2.2 Epidemiology

Peste des petits ruminants (PPR) is a viral disease that affects goats and sheep caused by peste des petits ruminants virus (PPRV), a negative sense single stranded RNA morbillivirus of the *paramyxoviridae* virus family (Gibbs, Taylor, Lawman, & Bryant, 1979) (OIE, 2016). PPRV has recently been renamed small ruminant morbillivirus (SRMV), however, it will henceforth be referred to as PPRV- its most commonly used name (Amarasinghe, et al., 2017) (Shabbir, Ul-Rahman, Zahid, & Munir, 2018). The virus family also contains other highly pathogenic viruses that affect a variety of species ranging from felines to humans (Libeau, Diallo, & Parida, 2014) (Baron, Diallo, Lancelot, & Libeau, 2016). PPRV is a small virus that ranges between 15,927-16,058 nucleotides in size (Bailey, Banyard, Dash, Ozkul, & Barrett, 2005) (Maes, et al., 2019). Based on sequence analysis, PPRV strains are divided into four lineages (I-IV); while all four are endemic in Africa, only PPRV of Type IV lineage is prevalent in Asia (Kumar, et al., 2014) (Parida, et al., 2015) (Rahman, et al., 2016). Type IV lineage has been reported to overwhelm not just Asia but also African countries possibly owing to stronger positive selection associated with the lineage (Albina, et al., 2013). PPRV, which is a highly contagious virus, was first characterized in 1942 in Côte d'Ivoire (Gargadennec & Lalanne, 1942). Within 15 years of the initial characterization, PPR was also reported in a number of other countries under different names including, 'Kata' (goat plaque) and 'peste des espèces ovine et caprine' (plague of ovine and caprine species), after more investigation, however, it was discovered these diseases were

one in the same (Mornet, Orue, & Gilbert, 1956) (Isoun & Mann, 1972) (Hamdy, Dardiri, Nduaka, Breese, & Ihemelandu, 1976) (Diallo, Barrett, Barbron, Subbarao, & Taylor, 1989). After its discovery in West Africa, PPR has since spread to almost all of Africa, the Middle East, Asia, and Eastern Europe (Shalia, et al., 1996) (Taylor, et al., 2002) (Fakri, et al., 2018). The most significant detection is perhaps its presence in the Middle East. Shortly after, it was detected in Turkey in 1996 which increased awareness of PPRV raising its relevance internationally because of the threat of disease spread to Europe (Ozkul, et al., 2002) (Kul, Kabakci, Atmaca, & Ozkul, 2007) (Al-Dubaib, 2009). Similar to CAPV diseases, PPR is prevalent in underdeveloped regions which often lack resources economically (Mariner, et al., 2016). While clinical systems are only observed in sheep and goats, other species such as pigs, camels, buffaloes, and cattle have been reported to undergo seroconversion when in contact with infected sheep and/or goats without detectable clinical disease (Gibbs, Taylor, Lawman, & Bryant, 1979) (Taylor & Abegunde, 1979) (Parida, et al., 2015). Despite the number of non-host animals able to be infected, there is no evidence of their active role in the spread of PPR (Mahapatra, et al., 2015). Additionally, there is experimental evidence of a European pig breed able to reproduce disease after inoculation with a PPRV Lineage IV strain isolated from goats. These pigs were also able to shed virus and transmit PPRV to other pigs through direct contact; this has not been further investigated (Schulz, Fast, Schlottau, Hoffmann, & Beer, 2018). Before it was properly characterized, PPR was wrongly diagnosed as rinderpest virus (RPV), an antigenically related virus of small ruminants and cattle (Taylor, et al., 2002) (Kumar, Barua, Riyesh, & Tripathi, 2017). The spread of PPRV outside of Africa and Asia into the Middle East was unavoidable as countries in that region including Saudi Arabia routinely import live sheep from PPR endemic countries likely without proper diagnostic tests performed prior to exporting in the originating country (Al-Dubaib, 2009). The economic effects of PPR are felt in the primary production system while pastoral communities act as a reservoir from which there are PPRV spillovers (Kitching R. , 1988) (Fournié, et al., 2018). In affected countries, and specifically in pastoral communities, PPR is considered one of if not the most important disease of ruminants due to the severe implication it has on infected animals (Mariner, et al., 2016). Additionally, small ruminants which are herd animals are in constant contact which exponentially increases their susceptibility to outbreaks (Anderson E. , 1995). PPR is an OIE notifiable disease that is believed to be the most important small ruminant infectious disease as

close to two billion small ruminants are in danger of exposure to PPRV (Diallo, Bataille, Lancelot, & Libeau, 2019).

1.2.3 Clinical disease

PPRV uses the H protein to attach to host cell surfaces using host receptors such as SLAM (lymphoid cell receptor) or nectin-4 (an epithelial cell receptor), this activates the F protein of PPRV to facilitate the release of PPRV DNA into the host cell cytoplasm (Parida, et al., 2015). PPRV causes acute respiratory disease often leading to death in affected small ruminants (Kumar, et al., 2014). The main clinical symptoms associated with the disease include fever and the presence of oral and nasal discharge; it can also result in erosive lesions, diarrhea, and pneumonia in severe cases (Parida, et al., 2015) (Mariner, et al., 2016). In pregnant animals, abortion may also occur (Kumar, Barua, Riyesh, & Tripathi, 2017). These symptoms result in a major problem in the diagnosis of PPR based on clinical signs due to the resemblance of symptoms to other small ruminant diseases such as foot and mouth disease (FMD), CAPV diseases, and bluetongue amongst others (Singh, et al., 2009). The clinical symptom onset can vary from 3 to 10 days while the incubation period is 4-6 days on average. From the first onset of fever, animals have been reported to die within the incubation period of 4-6 days (Kumar, et al., 2014) (Baron, Diallo, Lancelot, & Libeau, 2016). PPRV is lymphotropic and causes significant immunosuppression and lowered antibody response in infected animals (Parida, et al., 2015). It results in very high mortality and morbidity of up to 100% and 90% respectively (Pope, et al., 2013) (Baron, Diallo, Lancelot, & Libeau, 2016). It has been reported that goats generally have a higher mortality and morbidity compared to sheep, specifically the West African dwarf goat breed is the most compromised by PPRV (Lefèvre & Diallo, 1990). While PPRV and other morbillivirus infections result in a high death toll and/or morbidity, animals that survive an infection normally develop lifelong immunity against possible future infections (Kerdiles, et al., 2006). Due to the temperature sensitivity of the virus in external environments where PPR is endemic, close contact between animals is the main method of transmission. However, there are additional modes of transmission including shedding of virus in contaminated water, feed, and housing of animals (Braide, 1981) (Kumar, Barua, Riyesh, & Tripathi, 2017). Stemming from the successful eradication of rinderpest, there has been an initiative by international animal

health organizations to eradicate PPR by 2030, this can only be ensured through the use of safe and effective vaccination strategies (OIE, 2016) (Jones, et al., 2016). The eradication of PPR would not only benefit the subsistence and pastoral communities severely affected by the disease but it has also been shown that it would yield a net benefit of over 70 billion USD (Jones, et al., 2016).

1.2.4 Diagnosis and Therapeutics

The current OIE recommended diagnostic tools for PPRV include a competitive ELISA based on the N or H protein of PPRV and the gold standard virus neutralization test (Anderson & McKay, 1994) (OIE, 2016). There are also a number of serological tests available including an indirect ELISA based on the nucleoprotein (N) of PPRV (Ismail, et al., 1995), a sandwich ELISA based on the PPRV N protein (Saravanan, Sen, Balamurugan, Bandyopadhyay, & Singh, 2008), an immunocapture ELISA based on the PPRV N protein (Libeau, Diallo, Colas, & Guerre, 1994), and a haemagglutination-inhibition test (Raj, Nachimuthu, & Nainar, 2000). Virus isolation of PPRV has been reported as a possible alternative for diagnostics, however, reports have not been consistent. Marmoset-derived cell lines (B95a), primary lamb kidney cells, African green monkey kidney (Vero) cells, and Vero/SLAM cells have been used for virus isolation with Vero/SLAM cells becoming more widely used to grow morbilliviruses with a successful track record (Seki, Ono, Yamaguchi, & Yanagi, 2003) (Sreenivasa, Singh, Mondal, Dhar, & Bandyopadhyay, 2006). Vero-Nectin cells have also been demonstrated to be useful for PPRV isolation (Fakri, et al., 2016). There are also a number of molecular tests such as RT-PCR used routinely with serological assays to detect the presence of PPR (Devi, Das, Sharma, & Dutta, 2016) (Karim, et al., 2016). To date, no therapeutics are used to treat PPR infected animals. However, chemically synthesized small interfering RNAs (siRNA) have been proposed to target the N protein of PPRV to inhibit viral replication (de Almeida, Keita, Libeau, & Albina, 2007). In the case of PPRV eradication, the development of therapeutics may help to limit the spread of PPR as reported cases become fewer.

1.2.5 Vaccines

Prior to the development of live attenuated PPR vaccines, a live attenuated RPV vaccine was used to control the disease with success, however, this vaccine was eventually banned due to global efforts to eradicate RPV (Bourdin, Rioche, & Laurent, 1970) (Kumar, Barua, Riyesh, & Tripathi, 2017). It is believed that use of the rinderpest virus to control PPRV has had some negative effects such as increasing genetic diversity of PPRV (Muniraju, et al., 2014). The current means of control of PPR is through vaccination with live attenuated vaccines, the first of which was produced in 1989 after serial passages in Vero cells (Diallo, et al., 2007) (OIE, 2016) (Eloiflin, et al., 2019). Immunization with live attenuated PPRV vaccines administered subcutaneously has been reported to protect animals for over three years (Singh, et al., 2009) (Zahur, et al., 2014). To date, there are at least six live attenuated PPR vaccines in use (Diallo, Bataille, Lancelot, & Libeau, 2019). The most commonly used vaccine against PPRV is the Nigeria 75/1 strain, a wild-type isolate attenuated after 75 passages in cell culture (Taylor & Abegunde, 1979) (Eloiflin, et al., 2019). Although currently used vaccines are effective in their ability to confer protection against PPRV in immunized animals, they have been reported to be thermo-labile, requiring a cold chain (Braide, 1981) (Kumar, Barua, Riyesh, & Tripathi, 2017). The need to maintain the vaccines in a cold chain is cause for concern as PPR endemic countries may not have the resources to maintain that and/or the local climate raises challenges in maintaining the vaccine at ideal temperatures. This issue is somewhat remedied by the deployment of different lyophilization strategies to lengthen the half-life of live vaccines (Mariner, Gachanja, Tindih, & Toye, 2017). Another possible issue of continued vaccination with attenuated live vaccines is reversion. The high mutation rate of RNA viruses presents a real threat however low it may be for morbilliviruses (Eloiflin, et al., 2019). Furthermore, the use of live attenuated vaccines is restricted in non-endemic areas. To deal with this restriction, several inactivated PPR vaccines have been developed over the last decade, but, due to the need to vaccinate with two doses to induce immunity (Cosseddu, et al., 2016) and the lack of *in vivo* studies to monitor immunity (Ronchi, et al., 2016), these vaccines have not been licensed to be used in the field. The use of recombinant vaccines, may be useful in the future with respect to eradication due to limitations with the use of live attenuated vaccines in countries free of disease (Kumar, Barua, Riyesh, & Tripathi, 2017). The use of DIVA vaccines would aid in determining

the transmission of PPR in vaccinated animals as well as dead end hosts such as cattle and buffaloes in the rare case of a transmission of PPR back into small ruminants (Muniraju, et al., 2014) (Mariner, et al., 2016).

Possible alternative vaccines to use in the global effort to eradicate PPRV include recombinant vaccines, DNA vaccines, and virus like particles (VLPs). This is important because, ideally, a new vaccine would have DIVA capability that would allow previously endemic countries to gain disease free status (Kumar, Barua, Riyesh, & Tripathi, 2017). A number of recombinant vaccine vectors expressing PPRV proteins have been developed to aid in vaccine development efforts against PPRV. Of the number of available poxvirus vectors, CAPV has been used as a favorable vector to express PPR proteins and confer varying levels of dual protection (discussed in previous section). Fowlpox, another member of the *poxviridae* family, has also been used as a vector but with less desirable results in which very poor humoral and cell-mediated response was observed in small ruminants (Herbert, Baron, Batten, Baron, & Taylor, 2014). Most recently, a recombinant Newcastle disease virus expressing the H protein of PPRV has been shown to protect against a virulent challenge of PPRV (Murr, Hoffmann, Grund, Römer-Oberdörfer, & Mettenleiter, 2020). Conversely, replication-defective recombinant human adenovirus 5 expressing PPRV proteins was able to show promise due to complete protection observed in goats after virulent challenge (Herbert, Baron, Batten, Baron, & Taylor, 2014) (Kumar, Barua, Riyesh, & Tripathi, 2017). A DNA vaccine that has been developed as a potential vaccine against PPR codes for an antibody that resembles the hemagglutinin-neuraminidase (HN) protein. This vaccine which is stable and safe to administer was able to generate immune response against the PPRV H protein anti-idiotype specific antibody in sheep (Apsana, Isloor, & Shaila, 2015), however further data is lacking regarding its ability to confer protection against virulent challenge. Lastly, a subunit vaccine consisting of H, F, N and matrix (M) proteins co-expressed using baculovirus which form into VLPs has been constructed. The VLPs were shown to confer neutralizing antibodies and strong immunity against a virulent PPRV infection in goats (Yan, et al., 2019). Alternatively VLPs could be developed which only contain the H, F and M proteins which would allow this type of VLP vaccine to have DIVA capability. The use of VLPs while effective would require a boost to elicit a similar response to live attenuated vaccines and would result in a higher cost of production (Donaldson, Lateef, Walker, Young, & Ward, 2018).

Therefore, the ideal PPR vaccine would be a recombinant vectored vaccine with DIVA capability.

2. RATIONALE, HYPOTHESIS, AND AIMS

2.1 Rationale

2.1.1 Generation of a DIVA vaccine

Capripoxviruses (CAPVs) have caused and continue to cause large losses to the sheep, goat and cattle industry. They directly affect economies of many developing and developed countries. Due to the immune response generated by the animals to currently used vaccines, it is not possible to use a serological test to determine whether or not an animal has been vaccinated. Differentiating infected from vaccinated individual vaccines (DIVA) are a possible means to control and monitor the spread of CAPVs. Our initial aim involves the modification of two members of the CAPV genus to remove the CP25 gene to generate a DIVA vaccine. The prior development of a competitive enzyme linked immunosorbent assay (ELISA) based on CP25 antigen led to the sole use of the CP25 as the DIVA vaccine target. A secondary aim of the project was to show the essential nature of the CP25 gene that no longer makes it the ideal candidate to modify in generating a DIVA vaccine.

2.1.2 Generation of a bivalent vaccine

Peste des petits ruminants (PPR) is a viral disease of goats and sheep that is caused by a PPR virus (PPRV). Currently the main method of control is through vaccination using a live attenuated vaccine. Due to the prevalence of these diseases concurrently in afflicted countries, the use of a bivalent vaccine would be cost effective and advantageous due to the thermostability of a CAPV vector. The aim of this project is to genetically modify a live attenuated CAPV vaccine to express an antigenic protein from PPR.

2.2 Hypothesis

1. CP25 is a candidate gene to develop a capripoxvirus DIVA vaccine.
2. A bivalent vaccine to protect against capripoxviruses and peste des petit ruminants virus in sheep will be generated.

2.3 Aims

2.3.1. To generate a DIVA vaccine/determine the viability of CP25 as a DIVA candidate

2.3.1.1 Study the protective ability of the live attenuated Romanian vaccine of SPPV

2.3.1.2 Study the binding capacity of an in-house blocking ELISA antibodies to determine strategy in modifying CP25 gene

2.3.1.3 Attempt the isolation of pure SPPV and LSDV viral plaques expressing a partial knock out construct of CP25

2.3.1.4 Attempt the isolation of SPPV and LSDV viral plaques with full knockout of CP25 or homologous genes from vaccinia virus and swine pox virus

2.3.2. To generate a dual protective capripoxvirus vaccine expressing PPR H protein

2.3.2.1 Design a construct to remove the IL-10 homologous region of SPPV and replace it with the PPR H protein

2.3.2.2 Purify a pure SPPV viral plaque expressing PPR H and design molecular checkpoints to confirm isolation

3.MATERIALS AND METHODS

3.1 Viruses and cell culture

The live attenuated vaccines used were the LSDV Neethling (OBP) and the Romanian SPPV viruses. In some instances, a Nigerian SPPV was used as a positive control in conventional PCRs. An ovine testes cell line (OA3.Ts) was used to grow all CAPVs. VeroNectin (African Green monkey cell line) cells were used to grow PPRV. Cell growth media contained Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Infection media contained DMEM supplemented with 2% FBS and 1% penicillin/streptomycin. VeroNectin cell media was supplemented with 1 mg/mL Geneticin to allow selective growth of Vero cells expressing nectin receptor. Cells were seeded at a concentration 70,000 cells/cm² (80-90% on Day 2) and incubated at 37°C with 5% CO₂. An inverted light microscope with fluorescence capability was used to observe cells for cytopathic effect (CPE).

3.2 Animal experiment

5 sheep per group of the Rideau Arcott X breed were used. The sheep were all male and 2 months old. They were obtained from Canada Sheep and Lamb, Sarto, Manitoba. They were acclimatized to their environment for 9 days prior to vaccination. On day post vaccination (DPV) 0, they were either vaccinated by the intradermal route with 100uL of 10⁶ TCID₅₀ Romanian live attenuated SPPV vaccine virus or cell culture supernatant. Sheep were monitored daily for temperature and any clinical symptoms. At DPV21 all sheep were inoculated with 100uL of 10⁵ TCID₅₀ Nigerian SPPV administered by intradermal injection and monitored for 20-22 more days.

3.3 Virus Neutralization Test

On the first day, OA3.Ts cells were seeded at 70,000 cells/cm². On day 2, 96 well plates were used to perform serial 2-fold dilutions of sera starting with a 1/8 dilution using DMEM

supplemented with 2% FBS and 1% penicillin/streptomycin in duplicate to a total volume of 100uL per well. The diluted sera was not added to the last row of the 96 well plate to act as negative control. Positive (serum from previously infected animals) and negative control sera were also serially diluted to a total volume of 100uL per well. A dilution of virus (Kenyan LSDV strain) to a concentration of 1000 TCID₅₀/mL was then prepared in DMEM with 2% FBS and 1% penicillin/streptomycin. 100uL of virus was added to all wells with an exception of cell control wells. The virus and sera mixture plate was then incubated for 1hour at 37°C with 5% CO₂. After incubation, the virus and sera mixture (total of 200uL) was transferred to OA3.Ts cell plates after removal of existing media. The cells were then incubated at 37°C with 5% CO₂ for 7-10 days to observe CPE.

3.4 Competitive enzyme linked immunosorbent assay

Recombinant CP25 protein at 60ng/well was diluted in 0.05 Molar (M) carbonate-bicarbonate buffer to coat a 96-well plate overnight at 4°C. Following incubation the plate was washed 5X with phosphate buffered saline with tween (PBS-T). The plate was then blocked with 1X Sigma casein blocking bufferTM (SigmaAldrich) for 1 hour at 37°C with shaking. The plate was then washed 5X with PBS-T. 100uL of serum diluted 1:1 in 1X Sigma casein blocking buffer was then added to corresponding wells including control sera. The plate was incubated at 37°C for 1 hour with shaking then washed 5X with PBST. Following washing, 100uL monoclonal antibody (F117 G5-1 – NCFAD) was added at a 1/500 dilution in 1X Sigma casein blocking buffer and incubated for 1 hour at 37°C with shaking. The 96-well plate was then washed 5X with PBS-T. Then, 100uL of HRP goat anti-mouse IgG (KPL) at a concentration of 1:10,000 in 1X Sigma casein blocking buffer was added followed by incubation for 1 hour at 37°C. The plate was then washed for a final time 5X with PBS-T. 100uL of TMB ELISA substrate (Sigma Aldrich) was added and incubated for 10 minutes at room temperature. Finally, 100uL of 1M sulfuric acid (H₂SO₄) was added to stop substrate development; absorbance was read at 450nm using the SpectraMax® Plus 384 Microplate Reader (Molecular Devices).

3.5 Peptide Assay

Peptides and antibodies were obtained from Mimotopes™. 200uL of blocking buffer was added per well of a 96-well, streptavidin coated plate then incubated for 1 hour at room temperature (RT). The plate was then washed with PBS-T 4X. Prior to test, peptides were diluted in dimethyl sulfoxide (DMSO) according to the manufacturers manual. 100uL of previously diluted peptide solutions were added into their corresponding well positions on the plate and placed on a shaker for 1 hour at RT. The solution was washed 4X with PBS-T. 100uL of antibody (diluted in PBST/sodium Azide at 1:1000) was then added to each of the wells with peptides. The plate was placed on a shaker for 1 hour at RT. The solution was then washed 4X with PBS-T. Anti-mouse HRP antibody (0.5mg/mL) was diluted 1:2000 with conjugate diluent and 100uL was added into the wells followed by 1 hour incubation at RT for 1 hour. The plate was then washed 4X with PBS-T, 100 TMB (3,3',5,5'-Tetramethylbenzidine) was added to each well. The plate was shaken for 10 minutes then the solution was stopped with 1M H₂SO₄. Absorbance was read at 450nm using the SpectraMax® Plus 384 Microplate Reader (Molecular Devices).

Table 3.1 List of overlapping peptides used in the peptide assay

1 : SGSGMLVDVPRSGTETDYD	11 : SGSGKISIMTSMVSLITIT	21 : SGSGTESELNVYRSCKGIV	31 : SGSGNDWISDYLDGTWGED
2 : SGSGPRSGTETDYDESNF	12 : SGSGTSMVSLITITILAF	22 : SGSGNVYRSCKGIVYSGHC	32 : SGSGDYLDGTWGEDGNVLF
3 : SGSGETDYDESNFTAFTG	13 : SGSGLITITILAFFNNTC	23 : SGSGCKGIVYSGHCYTFNS	33 : SGSGTWGEDGNVLFKEKNQ
4 : SGSGESNFTAFTGSTIYG	14 : SGSGILAFFNNTCELNQF	24 : SGSGYSGHCYTFNSEPKSF	34 : SGSGGNVLFKEKNQELEAI
5 : SGSGTAFTGSTIYGYGLKS	15 : SGSGFNNTCELNQFNEHKQ	25 : SGSGYTFNSEPKSFNDAYD	35 : SGSGKEKNQELEAIDISDE
6 : SGSGSTIYGYGLSKKNIK	16 : SGSGELNQFNEHKQYFLKN	26 : SGSGEPKSFNDAYDDCEKK	36 : SGSGELEAIDISDEMRSYY
7 : SGSGYGLSKKNIKVKVL	17 : SGSGNEHKQYFLKNPNPTT	27 : SGSGNDAYDDCEKKNSELP	37 : SGSGDISDEMRSYYCVRSF
8 : SGSGKKNIKKVKLINFICI	18 : SGSGYFLKNPNPTTYSDDD	28 : SGSGDCEKKNSELPNNLM	38 : SGSGISDEMRSYYCVRSF
9 : SGSGKKVKLINFICISIM	19 : SGSGPNPTTYSDDDESEL	29 : SGSGNSELPNNLMNDWIS	
10 : SGSGINFICISIMTSMVS	20 : SGSGYSDDDTESELNVYRS	30 : SGSGSNNLMNDWISDYLDG	

3.6 Western Blot

Samples were prepared to load by adding 1uL dithiothreitol (DTT), 25uL Nu Page sample loading buffer, 40uL of sample (virus supernatant/resuspended pellet), and 34uL MilliQ water. Samples were denatured at 95°C for 15 minutes. They were then loaded onto Bis Tris Gels with 3-morpholinepropanesulfonic acid (MOPS) running buffer and ran at 120 volts (V) for 1.5-2 hours. Gels were then transferred onto polyvinylidene difluoride (PVDF) membranes then blocked with 5% skim milk in PBS-T. Membrane was blocked overnight at 4°C or for 1 hour at RT with shaking. Membranes were then washed 3X with PBS-T for 5 minutes each. Undiluted monoclonal antibody (produced in house, supernatant) was then added to the membranes and incubated for 1 hour at RT with shaking. The membranes were then washed with PBS-T X3 for 5 minutes each. HRP goat anti-mouse IgG (KPL) was added at a dilution of 1:1000 in PBS-T and incubated for 1 hour at RT with shaking. Membranes were washed with PBST 3X for 5 minutes each. Detection solution (KPL TrueBlue™) was then added for 5 minutes with shaking until bands were visible. The solution was then neutralized by adding milliQ water (mQH₂O) then imaged.

3.7 DNA Digestion

This was done using the DNA free kit from ThermoFisher Scientific™. Reactions were performed with a total volume of 25uL total per sample digested using pointed bottom 96 well plates. 0.1 volume of DNase I buffer and 1uL of DNase I were added to extracted genetic material. This mixture was then incubated at 37°C for 30 minutes. Following incubation, 0.1 volume of re-suspended DNase inactivation reagent was added. This mixture was incubated for 2 minutes at RT mixing after initial addition and 3-4X within the incubation period. The mixture was then centrifuged at 10,000 x g for 1.5 minutes then the supernatant was carefully removed and transferred into a fresh 96-well plate.

3.8 DNA Extraction

MagMAX™ DNA extraction

The Applied Biosystems MagMAX™ pathogen RNA/DNA Kit (ThermoFisher) was used. Working solutions of the buffers were prepared as follows. Working wash solution 1 was prepared 2:1 in 100% isopropanol, working wash solution 2 was prepared 1:4 in >97% ethanol. The bead mix was prepared by adding 1:1 vortexed RNA binding beads and lysis/binding enhancer. The working lysis/binding solution was prepared by 1:1 lysis/binding solution concentrate and 100% isopropanol. Solutions were then added to 5, 96-deep well plates as follows. 20uL of bead mix, 130uL of lysis/binding solution, and 55uL of sample was added to the first deep well plate. 150uL of wash solution 1 and 2 added to 2 deep wells each. 50uL of elution buffer was added to a 96 well elution plate. They were then placed in a MagMAX-96 Magnetic Particle Processor (ThermoFisher) under the script: 4462359-DW-50. For a smaller number of samples MagMAX™ Express-24 (ThermoFisher) was used.

Isolation of Intact CAPV DNA for sequencing

OA3.Ts cells were grown in 12-16 T150 flasks to 80% confluence. The OA3.Ts cells were then infected with the desired CAPV at an MOI of approximately 0.01 and incubated at 37°C with 5%CO₂ until CPE of approximately 80% was observed. Flasks were then freeze-thawed (-80°C/RT) 3X to lyse cells and release virus. The cell suspension from the flasks was then transferred to 50mL falcon tubes and centrifuged at 2500 rounds per minute (rpm) for 20 minutes at 4°C. The supernatant was then transferred to ultra-centrifuge tubes and centrifuged at 39,100 X g for 2 hours at 4°C. The pellets were re-suspended in 0.5 mL PBS per tube and the re-suspended pellets were combined. The pelleted virus was then layered on a 36% sucrose cushion (12mL cushion with 6mL of overlaid virus on top) in SW32 Ti compatible centrifuge tubes, PBS was used to top of the mixture to fill the centrifuge tube. The samples were then centrifuged at 25,800 rpm for 90 minutes at 4°C. The supernatant was discarded and the pellets were re-suspended in a total volume 1mL PBS. A sucrose gradient was then prepared in SW 41 Ti compatible centrifuge tubes by gently layering 3mL of 40% sucrose, 2.2mL of 36% sucrose, 2.2mL of 32% sucrose, 2mL of 28% sucrose, 1mL of 24% sucrose. The suspended virus was then added on top of the sucrose gradient with a PBS top up. The sucrose gradient was then centrifuged at 26,000 x g for 65 minutes at 4°C. The milky band between the 40% and 30% bands was then collected using a syringe then re-suspended in PBS in a SW41Ti compatible centrifuge tube. The pellet was then re-suspended in 250uL tris ethylenediaminetetraacetic (TE)

buffer and transferred into an Eppendorf tube. 250uL 2X pox virus DNA extraction buffer (0.02X Tris pH 7.6 (1M), 0.007X 2-ME, 0.08X NaCl (2.5M), 0.08X EDTA (0.25M), 2% Sarkosyl, 52% Sucrose) was then added to the re-suspended pellet and mixed gently then incubated at 4°C for 30 minutes. 50uL of 1mg/mL Proteinase K and 20uL of 1mg/mL RNase A was added to the solution and mixed gently then incubated overnight at 37°C. The next day, 500uL of phenol:chloroform:isoamyl (PCI) alcohol was added to mixture, mixed gently then incubated at room temperature for 15 minutes with gentle shaking. The tube was allowed to stand for a few minutes to allow phases to separate then centrifuged at 13,200 rpm in a tabletop centrifuge for 10 minutes. The aqueous (top) layer was transferred into a new Eppendorf tube. The PCI alcohol step was then repeated by adding an equal volume of PCI alcohol to the tube. The aqueous phase was then transferred to new tube and 0.1X 3M NaOAc and 2X volume of 100% ethanol were added to the tube. The Eppendorf tube, now containing precipitated DNA was incubated at -80°C for 1 hour or more. After refrigeration, the tube was centrifuged at 13,200 rpm for 30 minutes to pellet DNA. The supernatant was then removed, the pellet was washed gently with 70% ethanol, then transferred to a new Eppendorf tube and centrifuged at 13,200 rpm for 10 minutes to pellet DNA. The supernatant was removed and residual ethanol was allowed to evaporate (5-10minutes), then DNA was re-suspended in 150uL acid TE buffer.

3.9 E.coli Transformation

An Eppendorf tube of competent E.coli (TOP10) cells were thawed on ice for 10 minutes. 5uL containing 1 picogram (pg)-100 nanogram (ng) of plasmid DNA was added to the cell mixture. The tube was carefully flicked 4-5X to mix cells and DNA. The mixture was then placed on ice for 30 minutes. The cells were then heat shocked at 42°C for 20 seconds. They were then placed on ice for 5 minutes. They were then placed at 37°C for 60 minutes with vigorous shaking (250rpm). The cells were mixed thoroughly by inversion and flicking. 50-100uL of cells (or desired dilutions) were spread plated onto agar plates containing ampicillin or carbenicillin.

3.10 Plasmid Design

CP25 Partial knockout

The initial plasmid used to create CP25 knockouts consisted of a disrupted CP25 sequence with two selectable markers eGFP and gpt driven by P11

(CTTTAACTACTTGCATAGATAGGTAATTACAGTGATGCCTACATGCCGTTTTTTTGAA
ACTGAATAGATGCGTCTAGAAGCGATGCTACGCTAGTCACAATCACCACCTTTCATAT
TTAGAATATATGTATGTAAAAATATAGTAGAATTTTCATTTTGTTTTTTTTCTATGCTAT
AAATGAAT) and PE/L

(AAAAATTGAAATTTTATTTTTTTTTTTTGGGAATATAAATAAAGCTTGCT) promoters respectively. The P11 promoter was also included in the construct following the GPT gene to allow excision of the selectable markers during negative selection.

This disrupted sequence was flanked on either side of the selection markers for homologous recombination. The left flank:

ATTTCCCTTTGTTTCGACATATTTTCAATTTTTCTTTTGTTGTAATTAATGCATAAT
CGAGTTCTTCTGGAGTCGTTACTCTATTGACATGATCGGGCCATATATAACTATCATA
TGCAGAATTGTAAACAGGAGTAAATAAAAAATATATGTTTGTATTTTTCAACTAATGT
ACTAAATAAAGATAAAAGATATGCAGTTTTCCCTGATCCGGATCCGCCAACTAATGC
TATCCTAAATGGATTTGTTATTAACTATTTCTTAAAAAATGCTTTTCCTTGAACCTA
TTCATCATATTTATAATTACTGTTATTAGTTAATTTGGTATTATTAATAAATAAATGT
AGAAAAATAAGCCACAAATATCGAGACAATTAAACTTGTAACATAAAAAAAAAAAT
ACGTTACAAATAAAAGAACTTTAAAA

Right Flank:

AAAAAAAAAAAAATAATTATTATATTACTATAAATGAAGTCATTAAATAGACAAACA
ATAAATAAGATTAAACGTGCTTCTGCACCTACTGCTATTTTTGTACTGGTATTAAC
TTGTTAGTAGTATAGGTACTACAATACGATACAAAGATGAAGTATTTCCCTAATGCAT
GTAATAAAGGATGGGTACCATATGATGATAGCTGTTACTTAGATTCAAAGCTTCAAC
TTTCATTATATGGTGGTGTAAATGTTATGTAATAAGTATAATGCAAAGATACCTAATG
TTAGTATTAGACATTTAAGAGTGATCTCGTTAACTTATGGTAGACAGTTTTGGTATG
GACTCGAAAAAAAAAAAAATAATATATGGGTAGATGTAAATAGTAATAGCACTGTA
GATATGGATAAAAAACAGAACTTAGTAATA

CP25 Full Knockout

The full knockout and swap plasmids were designed to ensure removal of the CP25 gene alone or with the addition of CP26 gene removal. The full knockout plasmid contained both selection markers as the initial plasmid with the difference of the flanking region on either side containing DNA on either side of the CP25 gene without containing any of the gene itself.

Left flank:

```
CTTTGTTTCGACATATTTTTCAATTTTTCTTTGTTGTAATTAATGCATAATCGAGTT
CTTCTGGAGTCGTTACTCTATTGACATGATCGGGCCATATATAACTATCATATGCAG
AATTGTAAACAGGAGTAAATAAAAATATATGTTTGTATTTTTCAACTAATGTACTAA
ATAAAGATAAAAGATATGCAGTTTTCCCTGATCCGGATCCACCAACTAATGCTATCC
TAAATGGATTTGTTATTAACTATTTCTTAAAAAATGCTTTTCCTTGAACCTATTCAT
CATATTTATAATTACTGTTATTAGTTAATTTGGTATTATTAATAAATAAATGTAGAAA
AATAAGCCACAAATATCGAGACAATTAAACTTGTAACATAAAAAAAAAAATACGTT
ACAAATAAAAGAACTTTAAAAGAATTCGTTTAAACCTGCAGGACTAG
```

Right flank:

```
CTGAATCTGAGTTAAATGTTTATAGATCATGTAAAGGTATTGTTTATAGCGGACACT
GCTACACTTTTAACTCAGAACCTAAAAGTTTTAATGATGCATACGATGATTGTGAAA
AAAAAATAGCGAATTACCATCAAATAATTTAATGAATGATTGGATAAGTGACTAC
TTAGATGGGACGTGGGGAGAAGATGGTAACGTACTTTTTTAAAGAAAAAAATCAAGA
ACTTGAAGCTATAGATATAAGCGATGAGATGAGAAGCTATTACTGTGTAAGATCTTT
TTTTTAAAAAAAAAAAAAAAAATAAATTATTATATTACTATAAATGAAGTCATTAAATAGA
CAAACAATAAATAAGATTAAACGTGCTTCTGCACCTACTGCTATTTTGTACTGGTA
TTAACTATTG
```

CP25/123 Knockout/Swine Pox virus and Vaccinia virus swap

Swine pox and vaccinia virus orthologues of the CP25/CP26 genes which are the SPV119/120 and VACWR156/157 genes respectively were obtained from the national center for biotechnology information (NCBITM). The native promoter regions were retained, the construct was designed with GPT and eGFP selection markers as previously described. The inserted genes and selection markers were flanked on either side by regions surrounding the CP25 region on the left and CP26 region on the right. The Vaccinia virus construct included loxP regions on either

side of the selection markers instead of an extra P11 site to compare excision efficiency of loxP versus natural excision.

Left flank:

```
CTTTGTTTCGACATATTTTCAATTTTCTTTGTTGTAATTAATGCATAATCGAGTT
CTTCTGGAGTCGTTACTCTATTGACATGATCGGGCCATATATAACTATCATATGCAG
AATTGTAAACAGGAGTAAATAAAAATATATGTTTGTATTTTCAACTAATGTACTAA
ATAAAGATAAAAGATATGCAGTTTTCCCTGATCCGGATCCACCAACTAATGCTATCC
TAAATGGATTTGTTATTAACTATTTCTTAAAAAATGCTTTTCCTTGAACCTATTCAT
CATATTTATAATTACTGTTATTAGTTAATTTGGTATTATTAATAAATAAATGTAGAAA
AATAAGCCACAAATATCGAGACAATTAAACTTGTA ACTATAAAAAAAAAAATACGTT
ACAAATAAAAGAACTTTAAAA
```

Right flank:

```
TAGTAATAGCACTGTAGATATGAATAAAAACACAGAACTTAGTAATATAAAAAAAA
GTAGTAAAGGAGATATTAATGCATGTTATGTTTATAATTTGGCCAATTTAAAAATG
TGTCGTGTAATTACGTAAGTTATATAATCTGTGTTAAAAGGTTATATAATTGAAAAA
TGAGATTACCTAAATAGAGGAAAATGGATTTTGACTTCATTTTCAACAAAGACGAGG
ATGATATTTATACGTTAATAACAACCTTTAGGTGTATTAAAAATAAAAAAAAAAAGAA
ATATCAAAAGTTTGTAGTGAAC TAGACATTAATTTTATAGAAACATTAGGACCTTAT
AATGTAGTATCTTTAAATATACACCCATTTCTTAACAATTTTATAGAACAATCAAATT
TGATTA ACTGTTA
```

PPRH/IL10 Homologue KO

The whole genome sequence of the Moroccan strain of PPRV was obtained from the NCBI. The open reading frame for the H gene was taken and analyzed against the H genes for similarity against the Benin, Ghana, Jhansi, Nigeria, and Sierra Leone isolates of PPRV using GeneiousTM. A plasmid construct was then assembled using Geneious consisting of a P7.5 promoter upstream of the Moroccan H gene, two selectable markers (GPT, eGFP) driven by P11 and P-E/L promoters respectively. These regions were flanked on either side by surrounding regions of the IL10 homologue gene of the Romanian vaccine strain of SPPV. Left Flank:

```
CTTTGGAACAATCATCATGTGCAAAAATCCATTTCCCTCTGTTAGAAGTCCTCCAGG
TCCTTGAATTTTATAACAGCCAGTTTACATAATCCTACTTCGATTTCCCATGTATTAT
```

TTTCTTCTTCTGTGCATCTCTTTATTGATATTCCGCTTACAGTACTAGTAAAAATGAA
AAAAGTTAATGATGACGTATTGTGCGACACAAACCACCTCGATACTACTGAACTTTTT
TTTCTATTATTAATACTAAAACTTGAAAACAAACCTTTTTTTCCTCTCAAGGATTTT
CATATGATAGAAAACCTGAACTTTTTCTTTGTCTAAGGAGTTTATATTAACAAAAA
AAACAGATTTTTTGTGCGATGAGGTAATCATGAAGGATTCATTAACCCTATCATTTC
AAAAAACTTCAAAAAATAATTATATAGAGTAGTATTTACCACC

Right Flank:

TCTAACTTATAAACTACAACATTATAGTTTCTTTTTATGTCATAGTTAATATTATC
TTTTTTTATTCTTAAGTTACATGTATAAATACCACTATCATCATGTGTTGTGTTTTT
ATAACCAACTTAAGTTTGTATTTTGTATCCTATCATCGTATAACACTTTGTATTAT
TTTTTAAATAACTACTAATAAGGTCAATATCCATACAATGTACTATTGAATTAAG
TCCTTCGTTGAATCTGTAAGTAATTGTTTCTTCTTTATCCAAAAAAGTTAATTTTAT
AGACATTTCTTCACAAATATAAATTCCGTCATCAATATTTATAACAGGGTACAACC
ATAAGTTGTACCCTTGAAGCGTTTTCATTAGTTACTTCTATTTTTTTTAAAATTTTGT
AATCGTTATAATTTCTTACAAATGATAGAGAGTTAGTTTGAAACCATGTTATGTCA
TATGATG

The assembled construct DNA was then submitted to GenScript™ from where the plasmids were obtained.

3.11 Transfection

Lipofectamine™ 300 reagent (Invitrogen) was used to perform all transfections. OA3.Ts cells were grown in 6-well plates to a confluency of 80-90% in DMEM containing 10% FBS, 1% penicillin/streptomycin. Cells were then infected with CAPV at an MOI of 0.01 or 0.1 for 1 hour in DMEM containing 2% FBS, 1% penicillin/streptomycin and incubated at 37°C with 5% CO₂. For a transfection in one well, 7.5uL of lipofectamine™ was added 125uL of Opti-MEM™ media was mixed in an Eppendorf tube. Then a master mix of DNA was prepared by adding 125uL of Opti-MEM™ media, 5ug of plasmid DNA, and 10uL of P3000™ reagent in a second Eppendorf tube. The two tubes were then mixed together and incubated at room temperature for 10-15 minutes. Afterwards, the DNA-lipid complex (250uL) was added drop wise onto cells

after removing infection media. The cells were then incubated at 37°C with 5% CO₂ for 2 hours after which they were topped up with Opti-MEM™ media.

3.12 CRISPR

Infection with/without transfection was performed as previously described. CRISPRMAX™ Reagent Cas9 nuclease transfection protocol for synthetic gRNA was used to insert guide RNAs and Cas9 as follows. 125uL of Opti-MEM™ media, 6250ng of Cas9 nuclease, 1200ng of gRNA (CAAAUAAAAGAACUUUAAA, AAAAAAAAAAAUAAUUAUU), and 12.5uL of Cas9 Plus™ reagent were mixed in an Eppendorf tube. In a second tube, 7.5uL of CRISPRMAX™ reagent and 125uL Opti-MEM™ media were mixed. The mixture from the first tube was then added to the second tube and incubated for 5-10 minutes at room temperature. It was then added drop wise into previously infected or transfected cells. The cells were then incubated at 37°C with 5% CO₂.

3.13 Plaque purification

OA3.Ts cells were seeded on 6-well plates at 70,000 cells/cm² in DMEM containing 10% FBS and 1% penicillin/streptomycin and incubated overnight at 37°C with 5% CO₂. On the following day, fresh carboxymethyl cellulose (CMC) media was prepared by first dissolving CMC 2%(W/V) in mQH₂O. The solution was then autoclaved at 121°C for 15 minutes and cooled to 37°C to add the following ingredients: 1X DMEM, 2% FBS, 0.37% NaHCO₃, 0.025M HEPES, 1% 0.4g/L folic acid, 1% 200mM L-Glutamine, 1% 100mM sodium pyruvate, 1% penicillin/streptomycin. After the addition of the components, the overlay media was warmed to 37°C until cells were ready. To prepare for plaque purification, the viral sample was serially diluted by a factor of 10 in 5 wells of a 6-well plates with the last well acting as a cell control to a total volume of 1mL. The dilutions were done in DMEM containing 2% FBS and 1% penicillin/streptomycin directly on cell culture plates after growth media was removed. The infected cells were then incubated at 37°C with 5% CO₂ for 1 hour. After an hour, the viral dilutions were replaced with 3 mL of CMC media then placed back in the incubator for 6-7 days with CPE observed daily. Once isolated plaques with CPE were observed using an inverted light

microscope, individual plaques were marked visibly. A day before picking the plaques, 24-well plates were seeded with OA3.Ts cells to confluency using DMEM with 10% FBS and 1% penicillin/streptomycin. Individual plaques were then picked using a P200 micropipette by pushing tip through the overlay media and gently touching/scraping the infected cells on the bottom of the plate while simultaneously aspirating a small volume of overlay media into the tip. The picked plaque was then re-suspended in 250uL of fresh DMEM with 2% FBS and 1% penicillin/streptomycin. The re-suspended plaques were then used to infect cells by adding 200uL of each plaque to a well of the 24-well plate seeded on the previous day. The cells were infected for 1 hour at 37°C with 5% CO₂. After the incubation, the cells were topped up with 1mL of DMEM with 2% FBS and 1% penicillin/streptomycin. The cells were then incubated for 7-10 days observing daily for CPE. After the presence of CPE (>80%), cells were then freeze thawed 3X to release virus for downstream use.

3.14 Conventional PCR

DreamTaq™ Green DNA polymerase (Thermofisher) was used to perform all conventional PCR used. PCR reactions were done in a total of 25uL for agarose visualization and 50uL for PCR purification. The PCR mix for 25uL was prepared as follows: 2.5uL of 10X DreamTaq Green buffer (Thermofisher), 0.5 uL 10mM deoxynucleoside triphosphate (DNTP) Mix (Qiagen), 2.5uL of 10uM reverse and forward primer, 0.125uL of Dream Taq DNA polymerase (Thermofisher), 3uL of 25mM MgCl₂ (Thermofisher), 8.875uL of PCR grade water, and 5uL of sample. For each primer used they were optimized using 6 PCR conditions shown below. The PCR products were then loaded on 1% agarose gel in 0.5X Tris-Borate-EDTA (TBE) buffer at 100V for 30-50minutes based on the size of the gel prepared/level of separation required.

Table 3.2 List of CP25 primers used for conventional PCR to analyze presence of gene and knockout construct of the LSDV Neethling strain and SPPV Romanian vaccines

Gene	Forward (5'-3')	Reverse (5'-3')	Size (bp)
CP25	TACAGCATTCACAGGTTCCAT	AAAAGTGTAGCAGTGTCCGC	310
CP25KO	ATTTCACGAATCGCAACCGC	AGTGGAGAGGGTGAAGGTGA	987
CP25NC	TCCGGATCCGCCAACTAATG	TCCCACGTCCCATCTAAGT	829

Table 3.3 Primers used for conventional PCR to observe the presence of the PPR-H gene and IL10 homologue of the Romanian SPPV vaccine.

Gene	Forward (5'-3')	Reverse (5'-3')	Size (bp)
PPRH	CCGGTGGTGTATGAATCCCC	CCCAATTGGCCTCGTCATCT	689
IL10	AATGTGACGACGTTAGCTTT GA	AAAATATCAAACCTCTCCCATAG CCT	391

Table 3.4 Conventional PCR conditions used in analysis of gene editing

	Condition 1	Condition 2
Initial Denaturation	95C/3:00/1X	95C/3:00/1X
Denaturation	95C/0:30/35X	95C/0:30/35X
Annealing	50C/0:30	55C/0:30
Extension	72C/2:00	72C/2:00
Final Extension	72C/5:00/1X	72C/10:00/1X

Table 3.5 Conventional PCR conditions used in optimization of PCR

	Condition 3	Condition 4	Condition 5	Condition 6
Initial Denaturation	95C/3:00/1X	95C/3:00/1X	95C/3:00/1X	95C/2:00/1X
Denaturation	95C/0:30/35X	95C/0:30/35X	95C/2:00/40X	95C/0:30/40X
Annealing	50C/0:30	55C/0:30	55C/0:30	55C/0:30
Extension	72C/2:00	72C/0:30	72C/2:00	73C/1:00
Final Extension	72C/10:00/1X	72C/5:00/1X	68C/10:00/1X	72C/5:00/1X

3.15 PCR purification and sanger sequencing

PCR products were purified using the QIAquick™ PCR purification kit (Qiagen) as follows. 5X buffer PB was added to 1X PCR sample and mixed. The sample was then added to a QIAquick spin column in a 2mL collection tube. The column was centrifuged at full speed on a table top

centrifuge for 1 minute. The flow-through was discarded then 750uL of buffer PE was added to the column then centrifuged at full speed for 1 minute. The flow-through was discarded and the column was centrifuged for an additional minute. The column was then placed in an Eppendorf tube then 50uL of buffer EB was added then centrifuged for 1 minute at full speed after 2 minutes of incubation.

Sequencing of purified PCR products using the ABI Prism BigDye™ Terminator Cycle sequencing procedure was performed as follows. Firstly, PCR products underwent a PCR to amplify the target sequence. The sequencing master mix contained 13.5uL sterile water, 3.5uL 5X sequencing buffer, 1uL of BigDye Terminator v3.1 RR-100, 1uL of 5 pmol/uL primer, and 1uL purified PCR product (5-20ng). Final mixtures were then cycled at 96°C for 2 minutes X 1; 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes X 25, then cooled down to 4°C until use. The cycle sequencing reaction product was purified using the Qiagen DyeEx™ 2.0 spin kit. The spin column was vortexed to re-suspend the resin then placed in a 2mL collection tube. The column was then centrifuged for 3 minutes at 750 rpm. The spin column was transferred to an Eppendorf tube and the cycle sequencing reaction product was applied directly onto the slanted gel bed surface. The column was centrifuged for 3 minutes at 750 rpm to elute purified product. The Eppendorf tube was then placed on a vacuum spin for 30 minutes at 60 V-AQ with spin to precipitate the product. The precipitate was then re-suspended in 25uL Hi-Di formamide. Sanger sequencing was performed using the Applied Biosystems™ 3500xL Genetic Analyzer.

3.16 RT-PCR

The assay was designed using PPRH insertion plasmid (previous section). TaqMan™ Fast Virus 1-Step Master Mix (Applied biosystems) was used to design the PCR. The primers used were: forward (5'-3') – ATATTGCCGACGATGGACCT, reverse (5'-3') – ACCTTATGACGCCAAGGGAA, and probe – ACGATTGCATGCTCGCTCCTGG. Triplicates of serially diluted PPRH insertion plasmid DNA (10^{-1} - 10^{-7}) were added to 96 plates containing master mix at both concentrations of primer/probe to be tested. 5uL of sample was added to the PCR mastermix. ABI 7500 thermocycler (Applied Biosystems) was used for the reactions which

were cycled for: 50°C for 5 minutes, 95°C for 20 seconds, 40X 95°C for 3 seconds and 60°C for 30 seconds. Thermofisher 7500™ software was used to collect PCR data.

Table 3.6 RT-PCR conditions used to optimize PCR to detect PPR-H gene expression

	Condition 1 (uL)	Condition 2 (uL)
RNase-Free Water	8	5.5
4X TaqMan® Fast Virus 1-step MM	5	5
PPRHSeqF1 Primer (10uM)	0.8	1.8
PPRHSeqR1 Primer (10uM)	0.8	1.8
PPRHSeq Probe (10uM)	0.4	0.9
Total volume (μl)	15	15

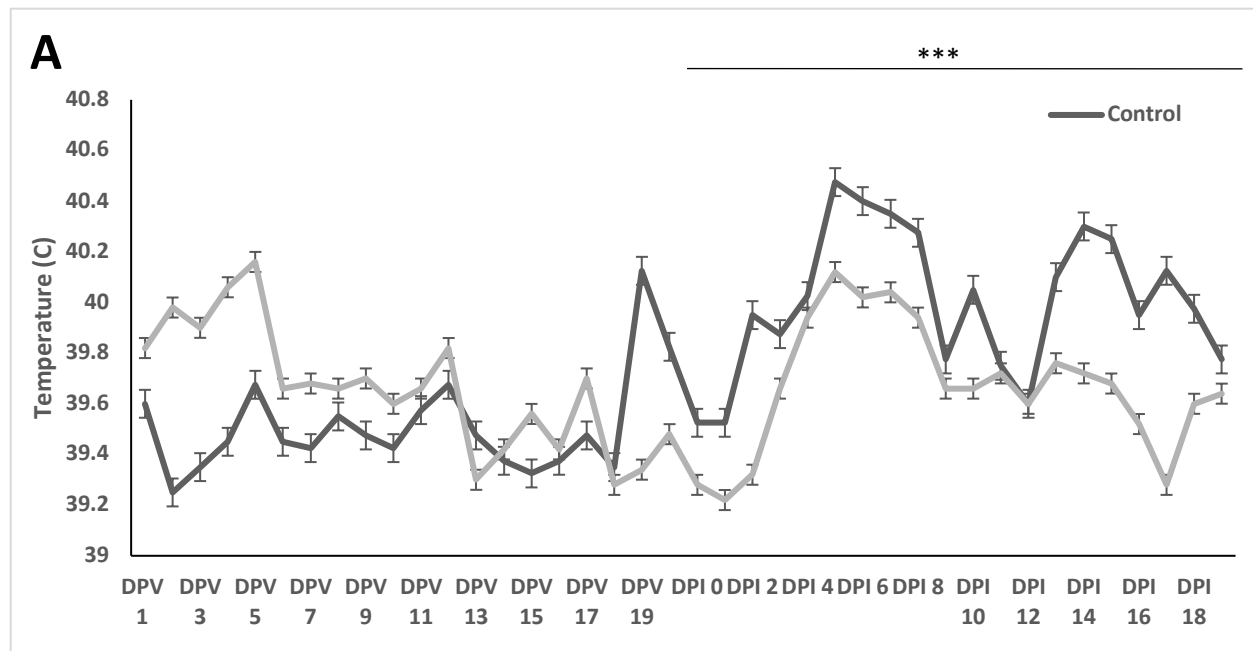
3.17 Statistics

Results are expressed as mean \pm SD when appropriate. Differences between groups were analyzed using a paired t test to determine statistically significant differences when appropriate using GraphPad prism 8. *P* values <0.05 were considered statistically significant.

4. RESULTS

4.1.1 The live attenuated Romanian virus (SPPV) confers full protection of immunized animals

To assess the protective ability of the Romanian SPPV vaccine in an experimental setting, groups of sheep (N=5) were vaccinated at DPV 0. The animals were all challenged with a virulent strain (Nigerian) of SPPV to monitor protective ability of the vaccine. Qualitatively (data not shown) and quantitatively (Figure 4.1A,B,C) it was observed that vaccinated animals were protected and showed the presence of neutralizing antibodies (Figure 4.1B). Vaccinated animals showed a significantly reduced fever at peak point of clinical symptom manifestation around DPI7 (Figure 4.1A). Additionally, the presence of neutralizing antibodies and detection of anti-CP25 antibodies at DPV 14 shows the ability of the vaccine to induce humoral immunity which is maintained for at least 42 DPV.



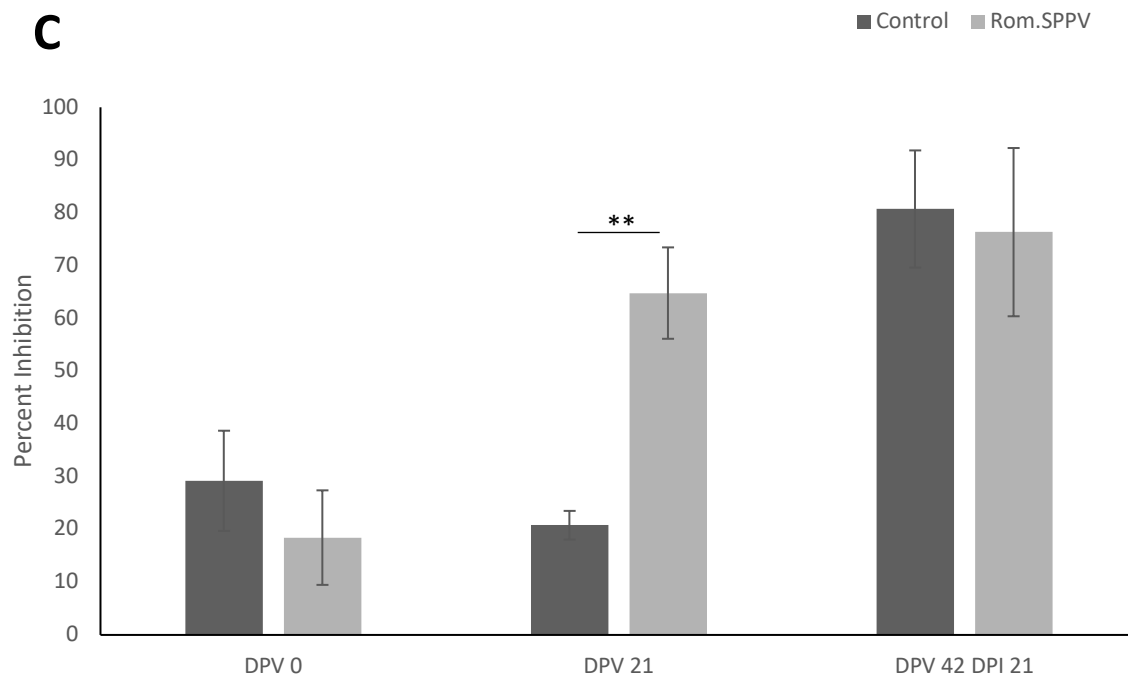
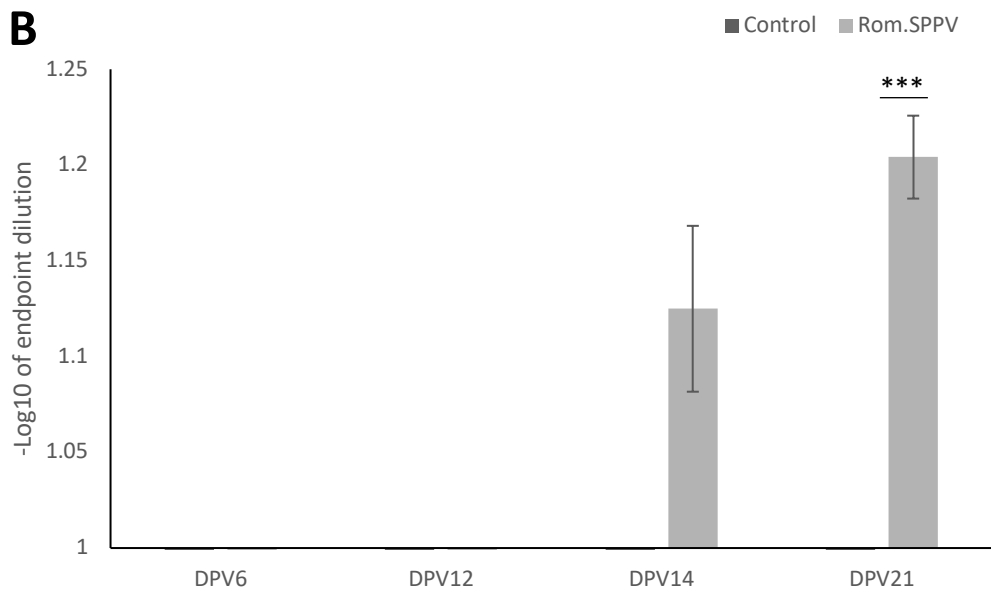
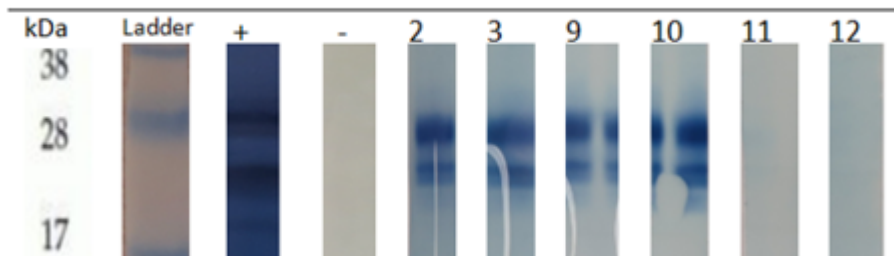


Figure 4.1.1 Vaccination with Romanian SPPV Vaccine confers full protection in sheep after challenge with a virulent SPPV strain. (A) Average temperature over time of control and vaccinated animals. Male Rideau Arcott X sheep were vaccinated intradermally with 10^6 TCID₅₀ Romanian SPPV vaccine strain or cell culture supernatant at DPV 0. Both groups were then challenged with 100uL of 10^5 TCID₅₀ Nigerian strain of SPPV intradermally and monitored daily for temperature and clinical symptoms. Temperature is expressed in mean \pm SEM, t test analysis for a paired two sample for means was done using excel for data from DPI1-19. (B) Virus neutralization assay was performed to observe the presence of neutralizing antibodies in vaccinated animals. There are neutralizing antibodies present from DPV 14. Data is presented as mean. (C) A blocking ELISA was also done to observe the presence of antibody response specific to the CP25 protein of CAPV. Percent inhibition of experimental animal sera was measured where a strong (>60%) inhibition in comparison to control positive serum reflects a strong antibody response. Data in B and C is presented a mean \pm SD. Data was analyzed for significance using GraphPad using a paired t test. **P<0.01, ***P<0.001.

4.1.2 Analysis of CP25 peptides to determine binding capability of monoclonal antibodies

To determine the binding ability of the in-house (National Centre for Foreign Animal Disease, NCFAD) produced monoclonal antibodies used in a CP25 blocking ELISA, Western blots and a peptide assay were performed. The peptide test was conducted with peptides consisting of overlapping amino acids covering the span of the CP25 protein totalling 38 peptides. This would allow for a targeted approach such as site directed mutagenesis to modify the CP25 gene to develop a DIVA vaccine, to minimize effects of gene alteration on function. However, it was found that antibodies utilized for the CP25 blocking ELISA (Ab11,12) were not able to bind a linear peptide implying their binding of a structural epitope. Both antibodies used in the blocking ELISA did not show strong binding to recombinant CP25 protein in Western blots (Figure 4.1.2A). Additionally, the lack of binding a linear epitope was further demonstrated the inability of both antibodies to bind a linear peptide in the peptide assay (Figure 4.1.2B).

A



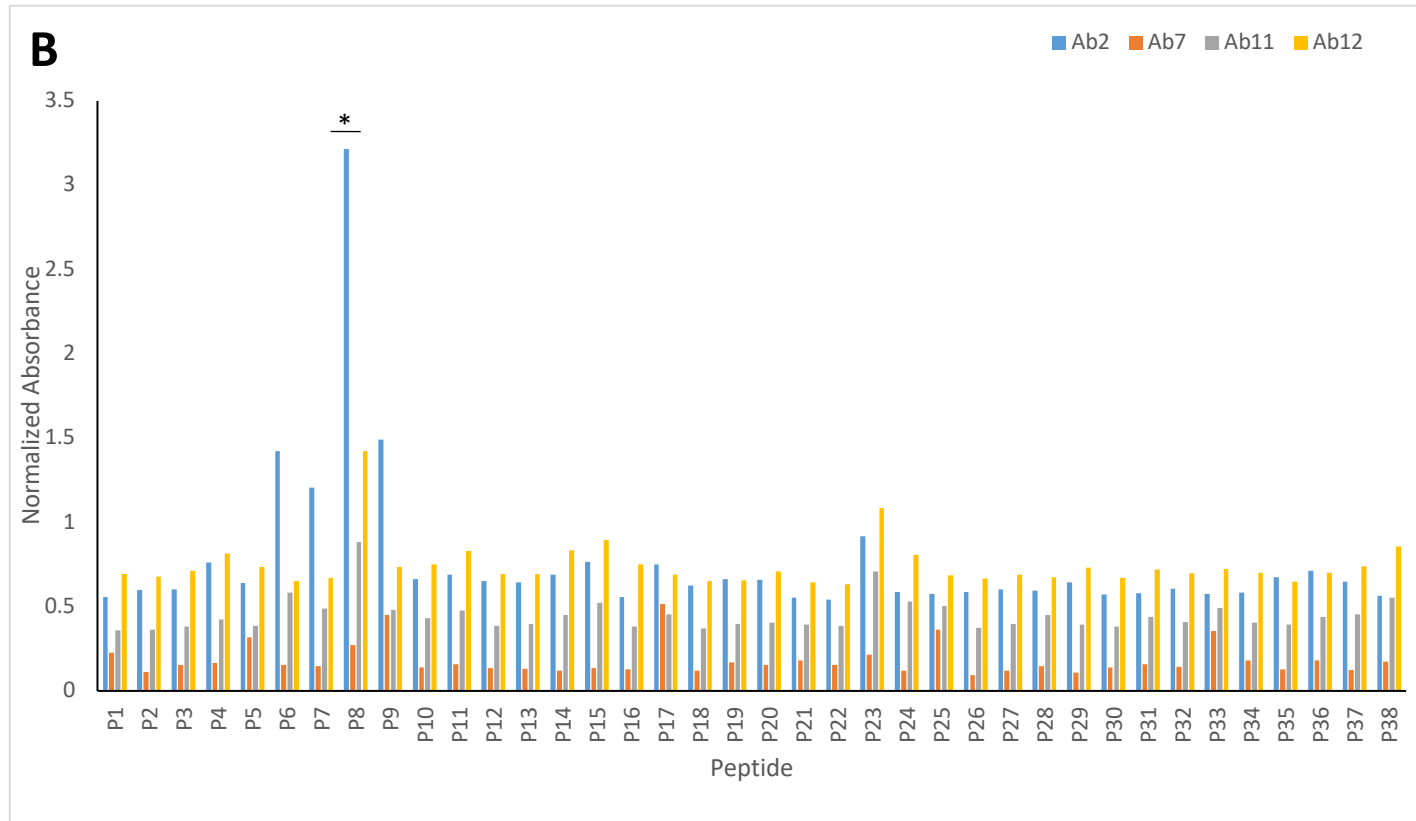
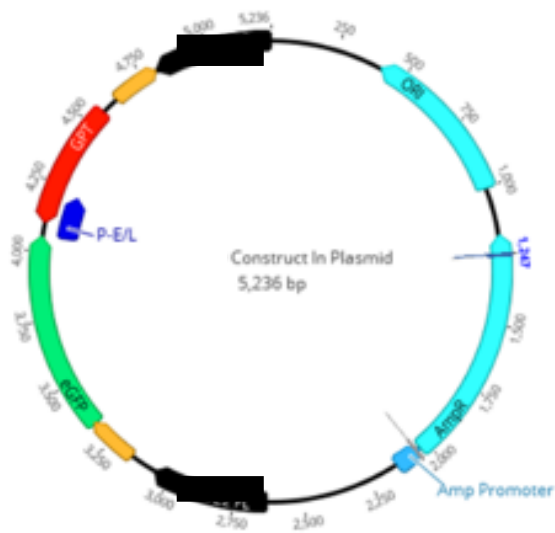


Figure 4.1.2 In-house monoclonal antibodies used in the blocking ELISA do not bind linear epitopes. (A) Western blot analysis. 1/50 diluted CP25 protein was used in sample preparation for Western blot. Polyclonal sheep serum was used as a positive control and cell supernatant as negative control. Antibodies 2, 3, 9, and 10 are strongly reacting monoclonal antibodies not used in the blocking ELISA that were produced at NCFAD. Antibodies 11 & 12 are used in the blocking ELISA. (B) Peptide binding assay. The peptide assay was conducted by using dissolved peptides (in DMSO) bound to a 96-well plate. Diluted antibodies that are used in the blocking ELISA (Ab11, Ab12) were not able to bind strongly to any peptides. Antibody 2 used as a positive control (reacted strongly in Western blot) was able to strongly bind to a peptide. Antibody 7 used as a negative control (not reactive in Western blot) also was not able to bind a peptide. Data was analyzed for significance using GraphPad using a paired t test. * $P < 0.05$

4.1.3 Transfection of OA3.Ts cells with CP25 partial knock out plasmid

Due to the binding of monoclonal antibodies used in the blocking ELISA to structural epitopes, disruption of the CP25 protein was necessary by partially knocking out the CP25 gene. This would be done through disrupting the structural integrity of the protein. OA3.Ts cells infected with SPPV and LSDV vaccines and transfected with a partial-knockout plasmid (Figure 4.1.3A) were both able to grow and express selective markers (Figure 4.1.3B).

A



B

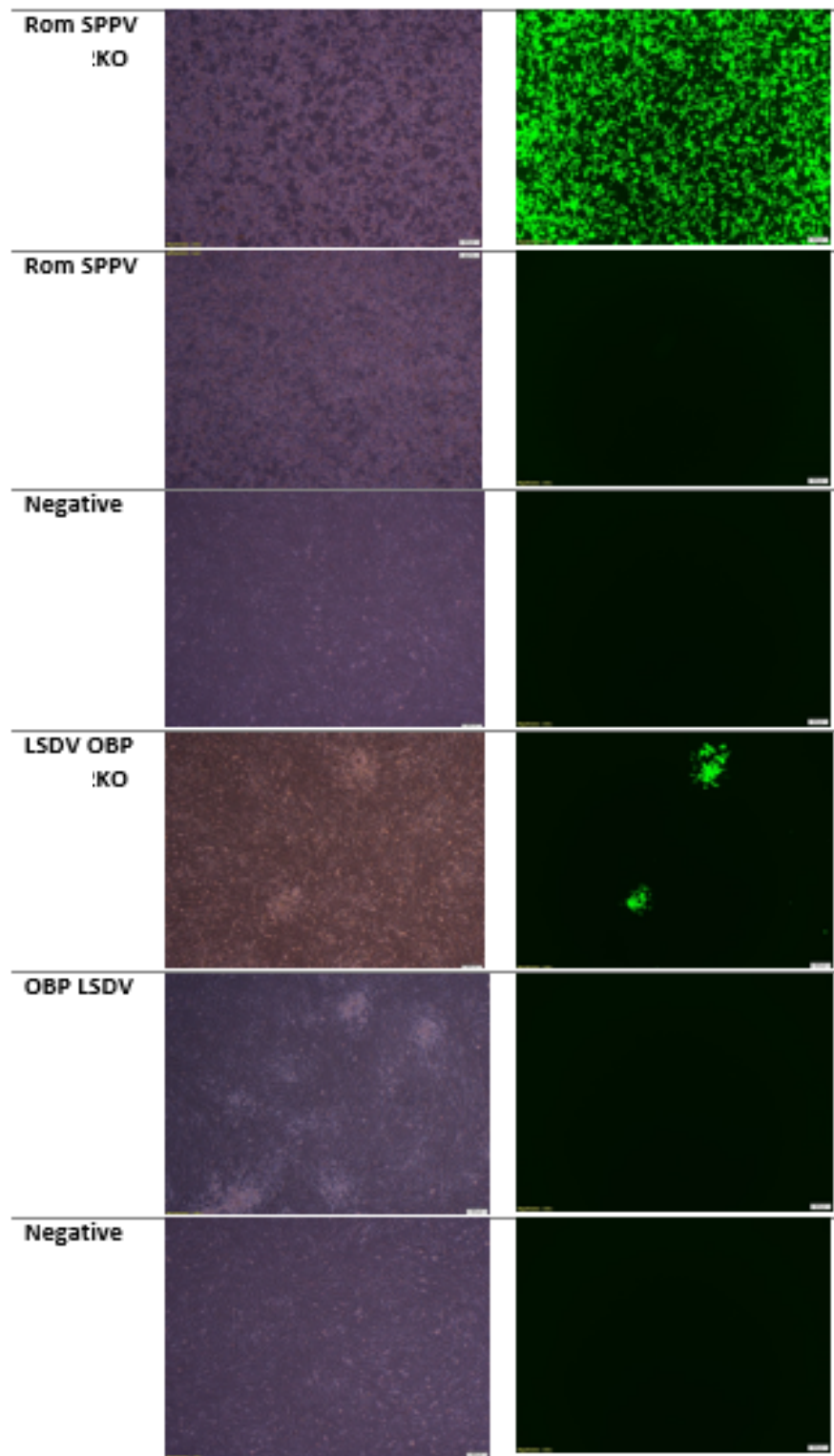


Figure 4.1.3 OA3.Ts cells were successfully transfected with a partial knockout CP25 Plasmid. (A) CP25 partial-knockout plasmid. (B) Transfected OA3.Ts cells at DPI7.

LipofectamineTM was used to transfect 5ug of plasmid DNA into OA3.Ts cells infected with Romanian SPPV vaccine virus or OBP LSDV vaccine virus at an MOI of 0.01. After 7 days, supernatant was collected following 3 freeze thaw cycles (-70°C/RT). Supernatant of transfected SPPV and LSDV was then used to infect OA3.Ts cells supplemented with gpt selection media. Virus was grown until GFP was observed (2-3weeks) in transfected SPPV and LSDV.

4.1.4 Mutant selection of SPPV and LSDV expressing mutant CP25

Numerous rounds of plaque purification were performed to isolate a pure viral plaque expressing mutant CP25. Pure mutant SPPV plaques could not be isolated after more than six rounds of purification suggesting a more essential role of CP25 protein. The presence of pure mutant LSDV plaques were predicted after conventional PCR analysis following the third round of plaque purification, however, after further molecular analysis (Sanger sequencing, next generation sequencing), it was observed that the wild type CP25 protein was present in all isolated plaques suspected to express mutant CP25 (Figure 4.1.4B,C). The difficulty in obtaining a pure mutant viral plaques in both viruses shows the possible essential nature of the CP25 gene. This observation lowers the likelihood of the successful development of a CAPV DIVA vaccine based on the CP25 gene.

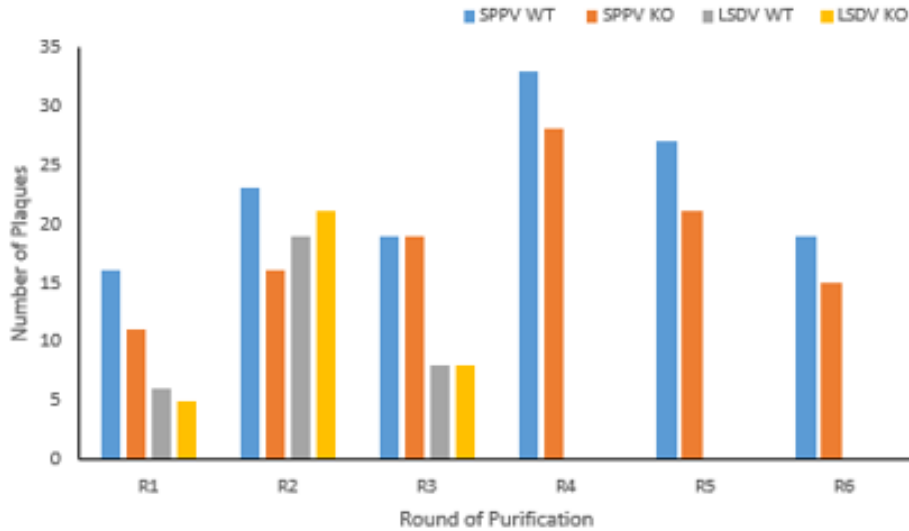
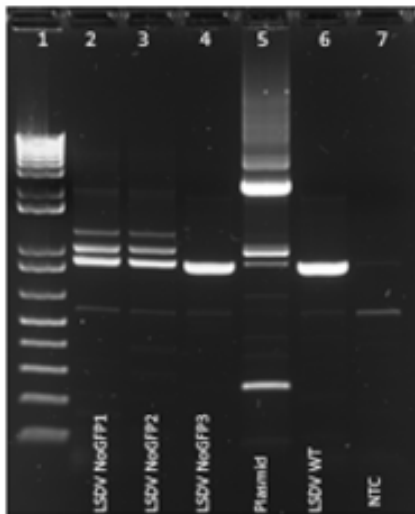
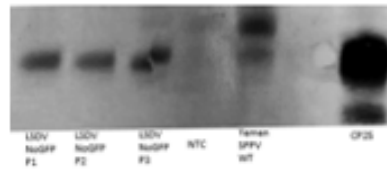
A**B****C**

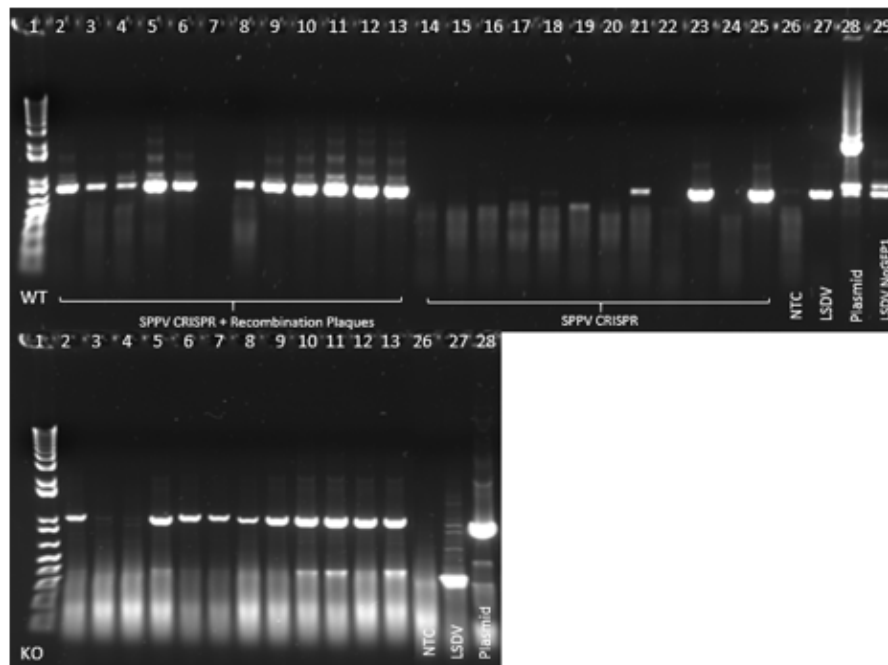
Figure 4.1.4 Inability to purify SPPV and LSDV plaques expressing mutant CP25 shows possible essential role of CP25 gene. (A) Plaque purification data of live attenuated LSDV OBP virus and SPPV Romanian virus vaccines. Following transfection with plasmid expressing selection markers to modify the CP25 gene, plaques expressing GFP were selected and purified. DNA from purified plaques was extracted and ran on two conventional PCRs, one (WT) to detect wild type CP25 DNA and the second (KO) to detect mutant CP25 DNA. SPPV plaques consistently had the presence of both wild type and mutant CP25 due to the presence of a mixed viral population. LSDV plaques that showed a presence of mutant CP25 without the presence of WT DNA were further purified. (B) Conventional PCR of LSDV plaques after further two rounds of purification and negative selection to remove GFP selection cassette. Plaque 1 and 2

show a similar pattern of a mixed population despite further rounds of purification whereas plaque 3 contains full WT DNA. 1kb Trackit™ (Invitrogen) DNA ladder was used. (C) Western blot of purified LSDV plaques to show the presence of WT protein. Western blot was ran as described previously with 1/50 dilution of recombinant CP25 protein used as positive control. Viral plaques were grown then underwent ultra-centrifugation to concentrate protein.

4.1.5 CRISPR/CAS9 transfection to evaluate effects of full knockout of CP25 gene

Due to the difficulty in isolating pure viral plaques containing mutant CP25, it was predicted that the gene may play an essential role. To test this, CRISPR/Cas9 was utilized to generate full CP25 knockout plaques. After transfection with guide RNAs directed at the gene and Cas9, it was observed that there were a number of plaques from both LSDV and SPPV that lacked the CP25 gene. These plaques, similar to what was observed with plaque purification of transfected virus, were not able to grow past the first round of purification. This provides further evidence of the important role of the CP25 gene in CAPV survival.

A



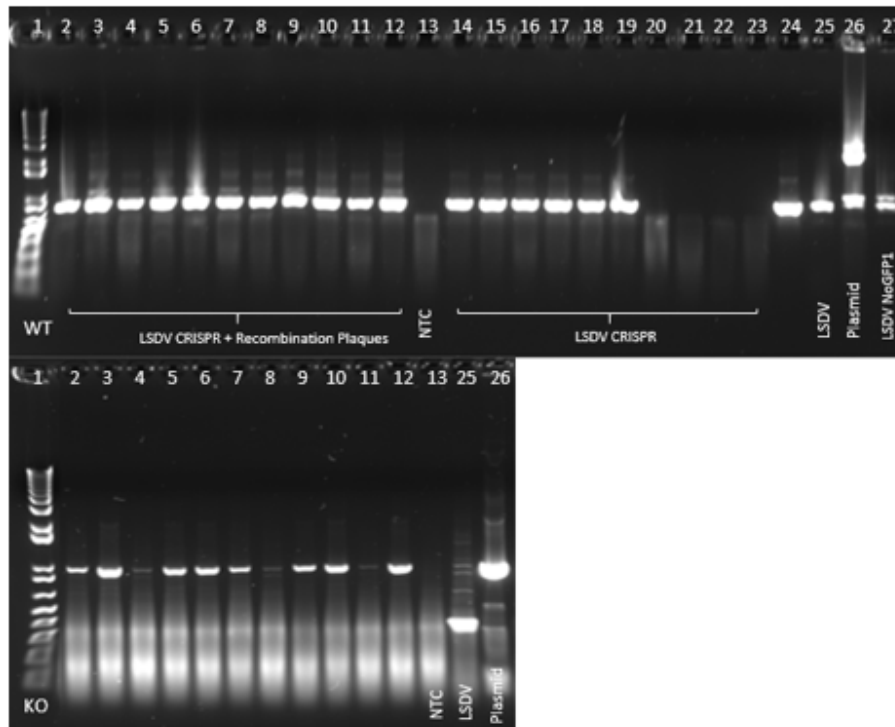
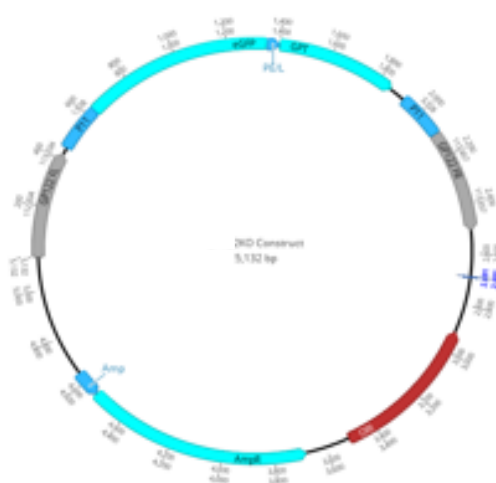
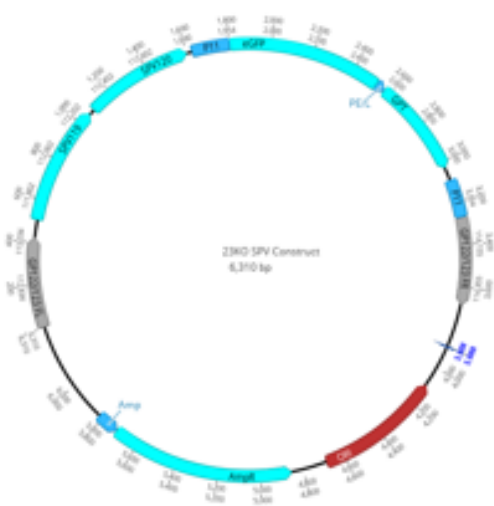
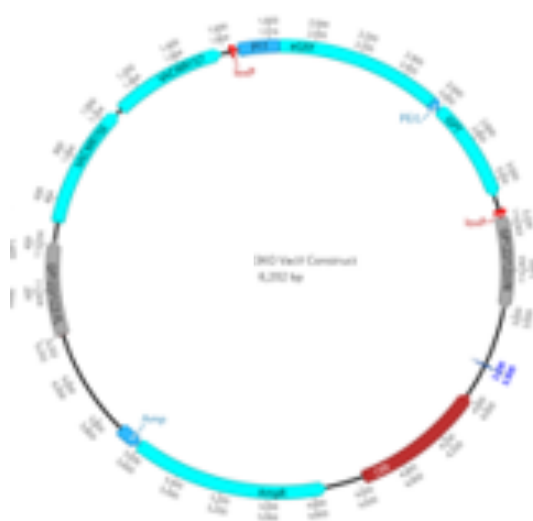
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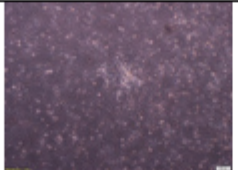

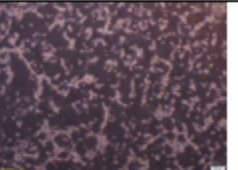



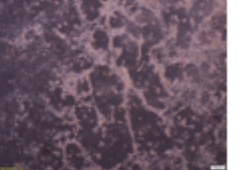
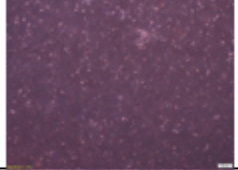
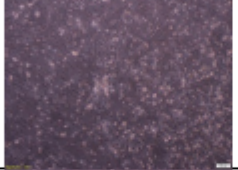
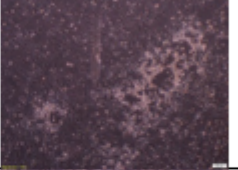


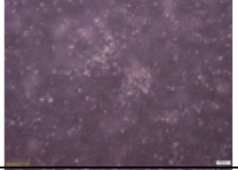
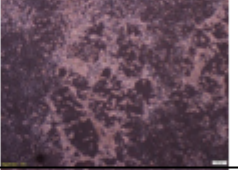






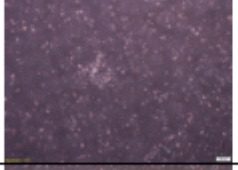
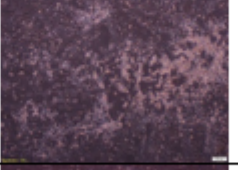









Figure 4.1.5 CRISPR/Cas9 resulted in full knockout of CP25 gene. Conventional PCR of (A) SPPV and (B) LSDV plaques purified after CRISPR transfection with or without plasmid. There were nine plaques that did not contain wild type CP25 DNA after CRISPR transfection, however, all plaques that underwent homologous recombination with plasmid contained wild type DNA. OA3.Ts cells infected with Romanian SPPV and OBP LSDV at an MOI of 0.1 were transfected with guide RNAs and Cas9 protein. This occurred with or without prior transfection with plasmid described in section 6.3.

4.1.6 Transfection of Romanian SPPV and OBP LSDV strains with plasmids containing CP25 homologues


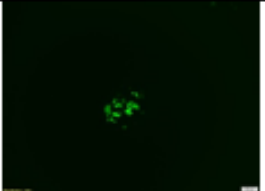
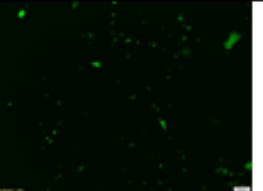
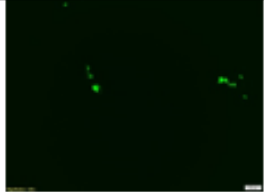
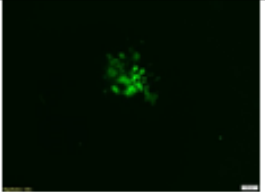
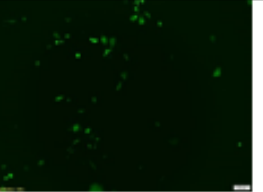


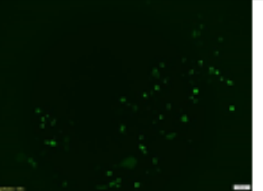
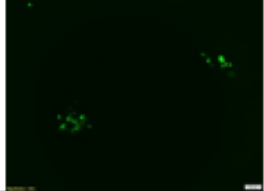
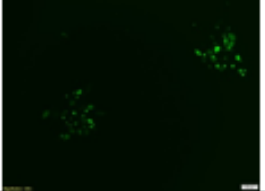
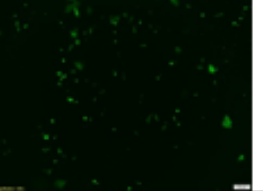
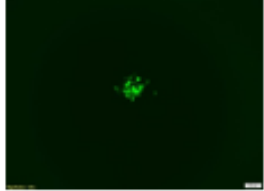
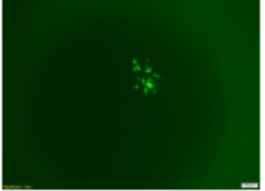
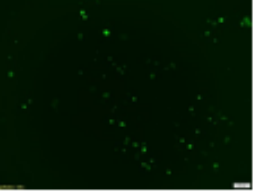
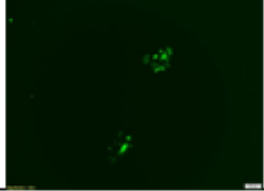
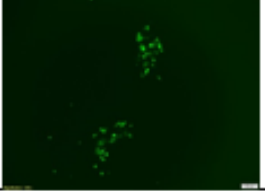
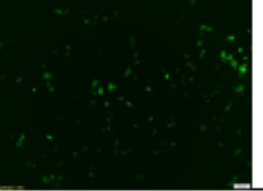
Working under the assumption of the importance of the CP25 gene, the last option to generate a DIVA vaccine while possibly maintaining the essential function of the CP25 protein was to rescue its function using genes from other poxviruses, due to the structural similarity of poxviruses. The poxviruses used were vaccinia virus (VACV) and swine pox virus (SPV). Using genome data from NCBI, plasmids were generated to result in a full swap of the CP25 and CP26 genes of CAPV. Both genes were selected for the swap because of previous knowledge based on studies done in VACV which suggested an interaction of CP25 and CP26 proteins to function properly (Personal communication, Dr. David Evans, University of Alberta). Upon transfection with the swap plasmids and a control (full CP25 knockout), presence of GFP plaques was observed. GFP observation proves the presence of some plaques containing fully knocked out or swapped CP25. Following the first round of selection which was attempted multiple times, no plaques were able to grow from any of the transfected groups. This evidence strongly suggests the importance of the CP25 gene which makes it no longer viable as a candidate for developing a DIVA vaccine.

A**B****C**

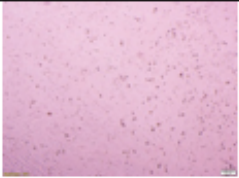
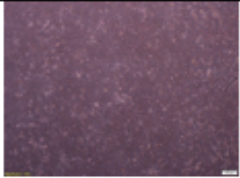
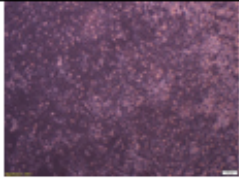
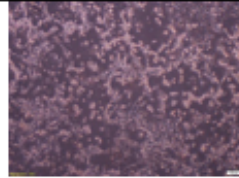
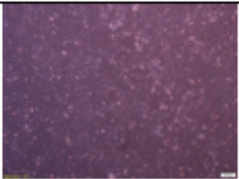
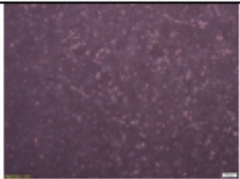
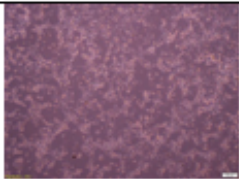
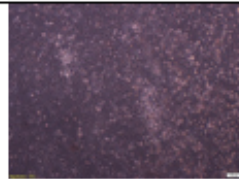


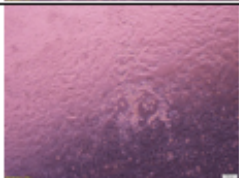

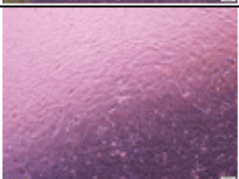
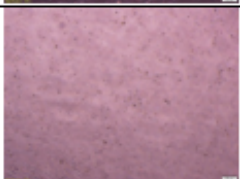


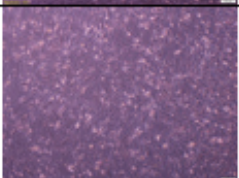
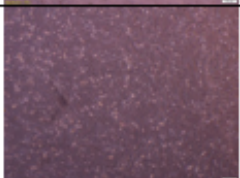






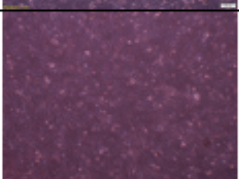
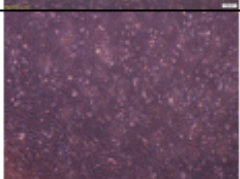
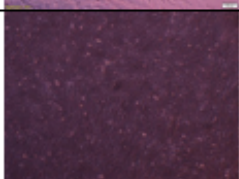
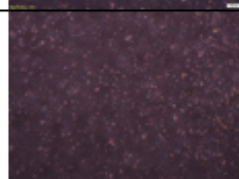
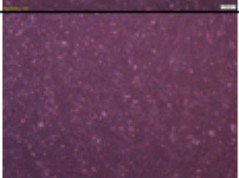
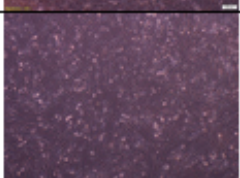
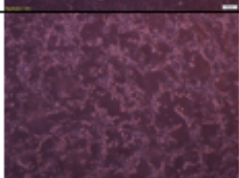
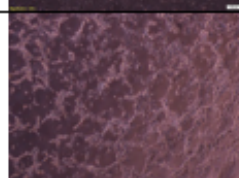
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Plasmid 1a				
Plasmid 1b				
Plasmid 2a				
Plasmid 2b				
Plasmid 3a				
Plasmid 3b				
Negative				
Positive				

E

	DPI 2	DPI 5	DPI 12
Plasmid 1a			
Plasmid 1b			
Plasmid 2a			
Plasmid 2b			
Plasmid 3a			
Plasmid 3b			

F

	DPI 2	DPI 5	DPI 12	DPI 13
Plasmid 1a				
Plasmid 1b				
Plasmid 2a				
Plasmid 2b				
Plasmid 3a				
Plasmid 3b				
Negative				
Positive				

G

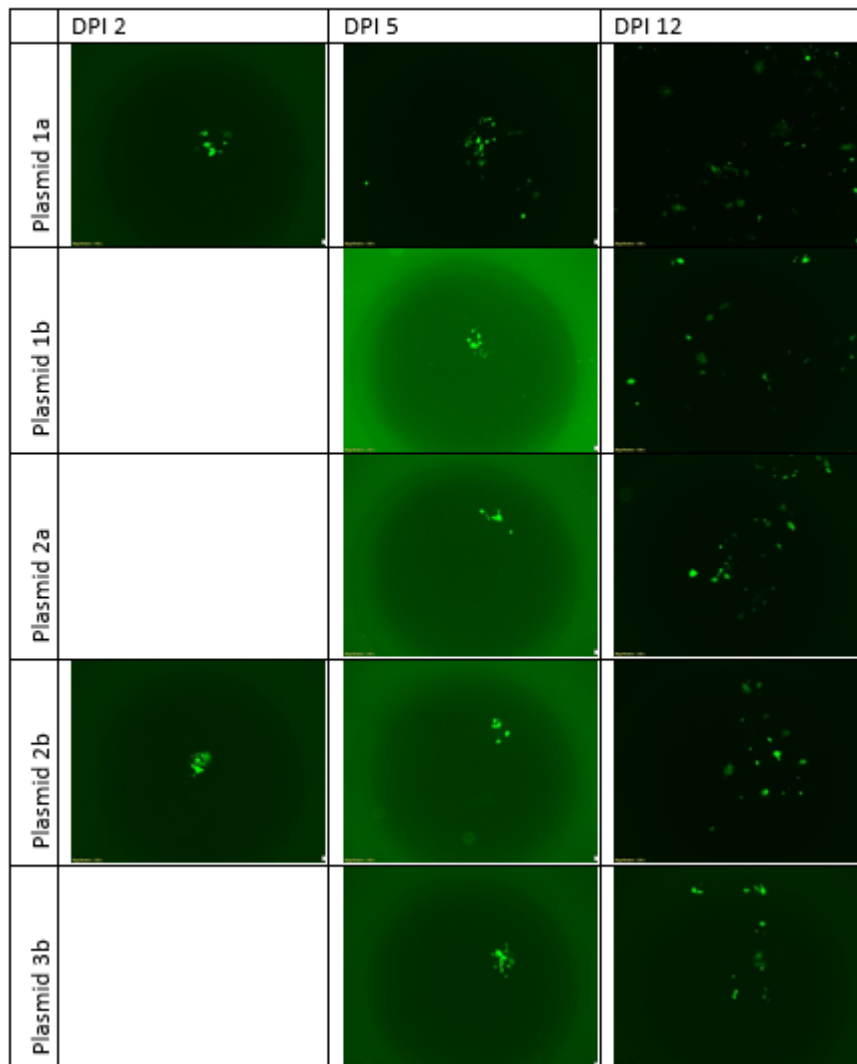


Figure 4.1.6 Transfection of SPPV and LSDV vaccine viruses with homologous genes from vaccinia virus and swinepox virus. (A) Full knockout plasmid of CP25 gene on LSDV and SPPV. (B) CP25/CP26 knockout plasmid with homologous swap from swinepox virus(). (C) CP25/CP26 knockout plasmid with homologous swap from vaccinia virus (). All three plasmids contain double selection markers (gpt and GFP) and allow for negative selection to remove selection cassettes. (D,E) OBP LSDV transfected with full knockout and swap plasmids (Plasmid 1.CP25 full knockout, 2.Swinepox virus swap, 3. Vaccinia virus swap). (F,G). Romanian SPPV transfected with full knockout and swap plasmids (Plasmid 1.CP25 full knockout, 2.Swinepox virus swap, 3. Vaccinia virus swap). OA3.Ts were infected with an MOI of 0.01 of SPPV and LSDV following transfection with 5ug of plasmid.

4.2.1 Transfection of Romanian SPPV vaccine with a plasmid containing the H protein from PPR

To create a bivalent CAPV/PPR vaccine, a plasmid was constructed containing the H gene from the Moroccan strain of PPR preceded by a poxvirus promoter. Flanking regions on either side of the H protein corresponding to the IL10 homologue gene of CAPV allowed for homologous recombination with the homologue gene to insert the H gene.

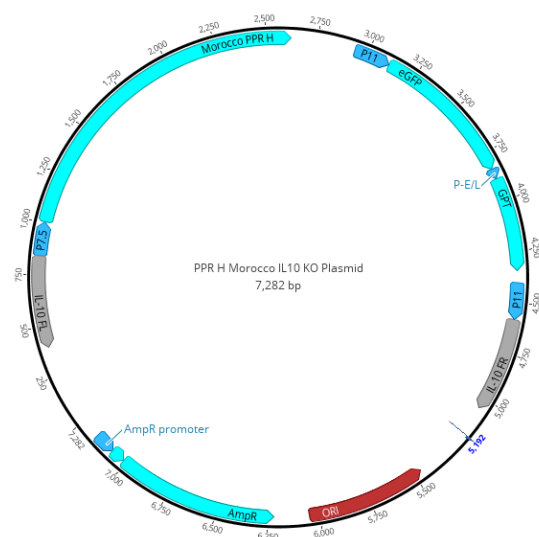
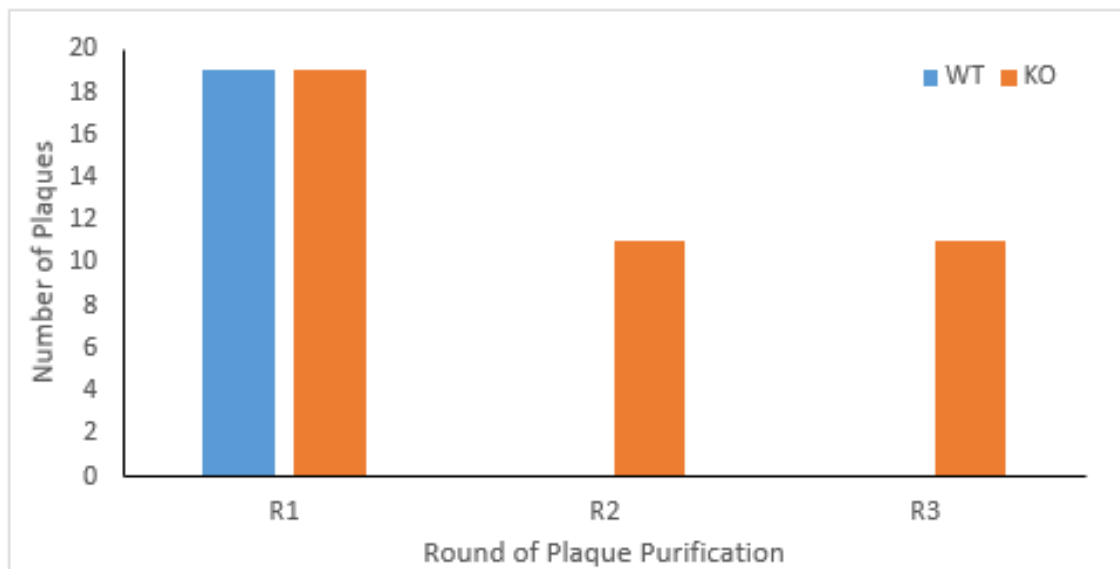


Figure 4.2.1 PPRH/IL10KO plasmid. The H protein of the Moroccan PPR virus was obtained from NCBI. Nucleotide alignment was performed with six other strains of PPR (Benin, Ghana, Jhansi, Nigeria, Sierra Leone) which resulted in >90% sequence similarity. The plasmid was constructed using Geneious™ by inserting a poxvirus (P7.5) promoter, two selection markers (gpt, GFP), and SPPV IL10 homologue regions flanking either side of the gene/selectable marker cassettes.

4.2.2 Romanian SPPV plaques expressing PPR H were successfully plaque purified

Upon transfection with plasmid (Section 4.2.1), SPPV plaques were purified using GFP and gpt selection. Positive selection involved the use of gpt selective media while picking plaques expressing GFP. Plaques with the H gene were present from the first round of purification. After the second round of purification, there were no viral plaques present containing the IL10 homologue region (no wildtype SPPV). Following this, plaques were negatively selected to remove the selectable marker cassette. A single viral plaque was obtained that successfully recombined the cassette out of its DNA.

A



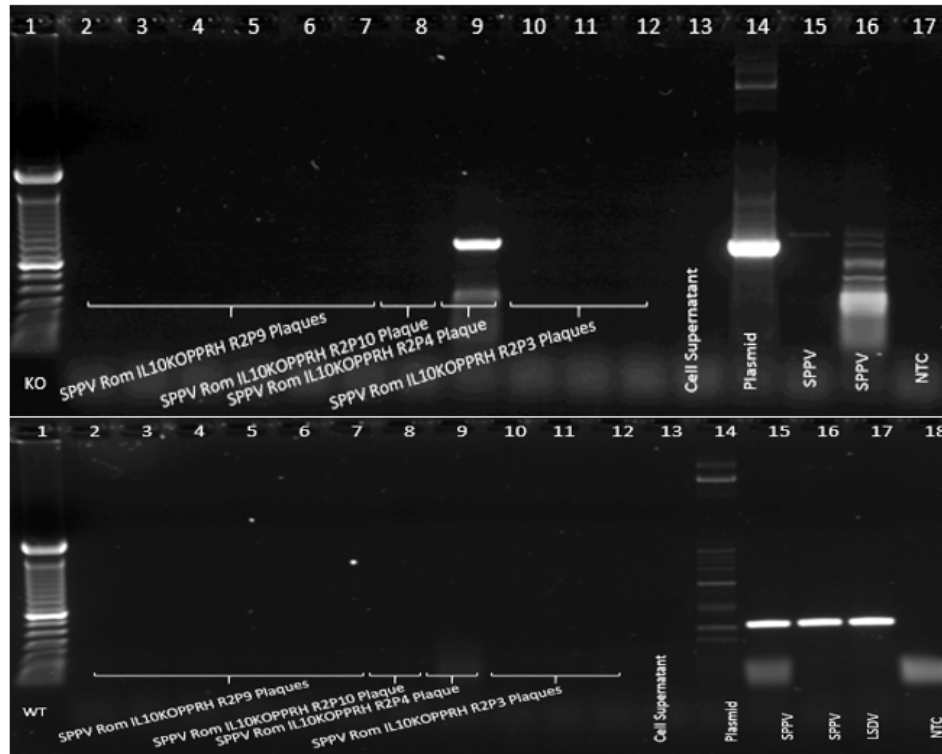
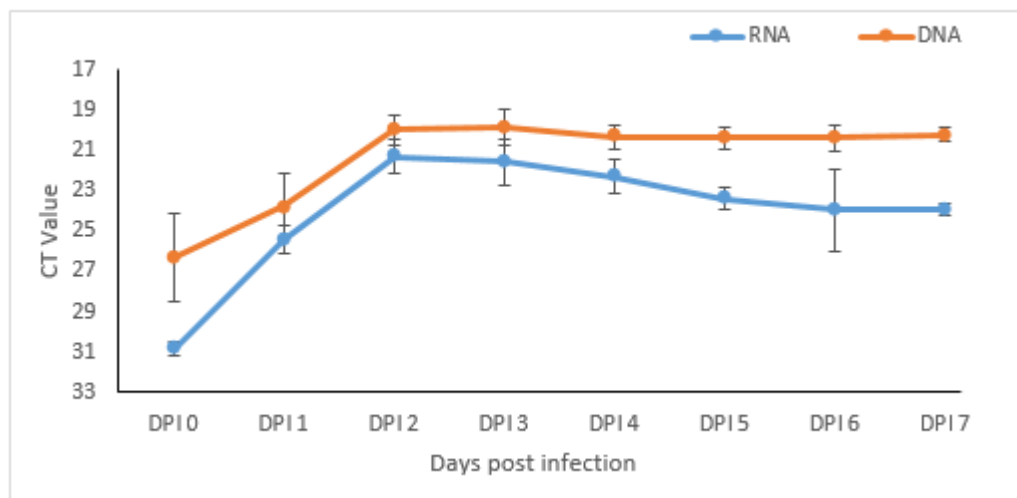
B

Figure 4.2.2 Purification of Romanian SPPV expressing PPRH A) Tabulated conventional PCR data of positive selection of Romanian SPPV Plaques expressing PPR H. After transfection, plaques were grown on gpt selection media then selected for using an inverted light microscope under UV light. Plaques with GFP were then further purified for two more rounds. (B) Conventional PCR of negatively selected Romanian SPPV plaques expressing PPR H. Positively selected plaques were grown on non-selective media (DMEM, 2% FBS, 1% penicillin/streptomycin). Non-GFP plaques were picked and grown to then examine the presence of the PPR H gene without the presence of wildtype virus.

4.2.3 Real-time PCR shows time course expression of PPR H gene

A single plaque of Romanian SPPV was selected showing PPR H gene presence with out GFP. This plaque was grown further to determine mRNA expression of H at numerous time points. An RT-PCR was developed to test the time course expression of the PPR H gene in SPPV using plasmid (Figure 4.2.1). The RT-PCR data shows mRNA expression beginning on DPI 1 with a peak at DPI 2.

A



B

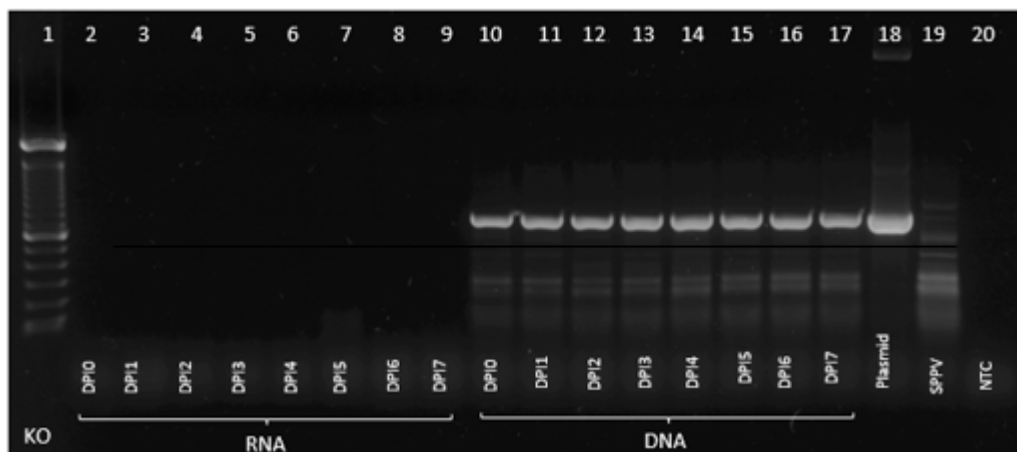


Figure 4.2.3. PPR H gene is successfully expressed on Romanian SPPV (A) Real-time RT-PCR data of PPRH DNA and mRNA from plaque purified Romanian SPPV. Positively selected plaques were subjected to negative selection to remove GFP/gpt selection cassette. A single plaque was obtained which was then grown further in OA3.Ts cells. Cells were infected at DPI 0 with the grown plaque in triplicates and a plate was frozen daily. DNA/RNA was extracted from after three rounds of freeze/thawing at -70°C. An aliquot of the extracted material was then subjected to DNase digestion. (B) Conventional PCR of DNase digested extracted genetic material. There was no contamination of RNA samples with DNA.

5. DISCUSSION

5.1 Role of CP25 in CAPV

Both vaccine and field isolates of CAPVs are not well characterized on the protein level. This lack of characterization has often led to the use of other well characterized poxviruses such as VACV, a homologous virus, as a point of reference in deducing the essential functions of genes, viral replication, and other basic information regarding CAPVs. In the attempt to generate a CAPV DIVA vaccine, numerous approaches were taken, however, the probable essential nature of the CP25 gene in the LSDV OBP Neethling vaccine virus and Romanian SPPV vaccine virus has prevented the generation of a CAPV DIVA vaccine lacking CP25 protein. Due to the development of a blocking ELISA using recombinant CP25 protein as the companion diagnostic test, CP25 was the only gene considered in the generation of a DIVA vaccine. Firstly, the binding capability of monoclonal antibodies used in the CP25 blocking ELISA was analyzed. This allowed for the determination of whether the antibodies bound structural or linear epitopes. Using western blots and a peptide assay, it was shown that the antibodies were not able to bind linear epitopes. Because they were found to bind structural epitopes, the possibility of site directed mutagenesis of CP25 was not an option. Site directed mutagenesis allows for alteration of one or more amino acids through nucleotide modification (Carter, 1986). Ideally, this system would prevent the binding monoclonal antibodies while minimizing the structural and functional effects on the CP25 protein itself. A partial KO plasmid was then constructed to essentially prevent transcription/translation of the CP25 protein while also leaving the downstream regions of the DNA intact to allow transcription of adjacent genes such as CP26, another structural protein. Numerous rounds of plaque purification were performed after transfection with a partial KO plasmid for both LSDV and SPPV vaccine viruses used. The inability to isolate a pure viral plaque with mutant CP25 gene was indicative of the important role the CP25 protein plays in CAPV survival. In previous reports and success in generation of other recombinant CAPV, pure mutant viral plaques are often isolated after three rounds of plaque purification (Wallace, Weyer, Nel, & Viljoen, 2007) (Boshra, Cao, & Babiuk, 2016). In the case of SPPV, despite the presence of GFP after numerous rounds of purification (>6), PCR data did not show the presence of plaques without WT DNA. This was also observed in LSDV, however, viral plaques were not

able to be isolated through positive selection past the third round of purification. The observation of GFP without the presence of mutant plaques is possible due to trans expression of GFP by CAPV polymerases resulting in a much smaller number of recombinant virus present relative to the amount of GFP expression (Boshra, Cao, & Babiuk, 2016). The final attempt in the construction of a DIVA vaccine, as a result of the difficulty in removing CP25 was a swap of the gene from orthologous poxviruses. The use of SPV and VACV genes to replace CP25/123 in CAPV was also demonstrated to not be able to rescue the virus. This could explain a host range specific function of CP25/123 in CAPV. In VACV, the equivalent gene to CP25 is A33R, a well characterized outer membrane protein. To confirm that the CP25 gene is essential gene, one copy of the CP25 gene can be placed in a non-essential gene in a different region of the virus and then this virus can be used to delete the CP25 gene in the original location. If this gene deleted virus can be made it would strongly suggest that the CP25 gene is an essential gene.

Following analysis of chordopoxvirus genes, it was proposed that conserved genes may be a relatively correct predictor of essential function in virus life cycle (Upton, Slack, Hunter, Ehlers, & Roper, 2003). Furthermore, it was observed that chordopoxviruses specifically had a much higher ratio of conserved genes that are involved in morphogenesis than genes involved in DNA/RNA synthesis; this phenomenon is opposite to what is observed in other poxviruses where replicative genes are conserved at a higher rate (Upton, Slack, Hunter, Ehlers, & Roper, 2003). Because of the higher probability of conservation of genes in morphogenesis in chordopoxviruses, a logical line could be made that would imply a stronger conservation of these genes in more species specific viruses such as SGPV and LSDV. This argument provides a foundation for the inability in removing the CP25 gene from both LSDV and SPPV. The orthologous gene in VACV,

. This gene, which is highly conserved in all orthopoxviruses, a genus in chordopoxvirinae, has been shown to have an important function .

In previous VACV studies, an deletion mutant could not be generated in VACV. This correlates to the difficulty in generating a deletion mutant in SPPV and LSDV.

Experimental results which reflected similar findings to studies in VACV suggest a functional homology between CP25 and . Although both proteins most likely play a similarly essential role in their respective virus, the species specific nature of CP25 is clear from the results of the orthologous virus swap experiment. Morphogenesis and egress, a role has been shown to have, are known to be host specific and rely on host proteins and membranes; this provides further explanation as to why a rescue experiment was not successful (Upton, Slack, Hunter, Ehlers, & Roper, 2003).

5.2 Bivalent CAPV vaccine expressing PPR H

PPR is a transboundary animal disease with significant socio-economic repercussions in endemic countries resulting in a global loss of over a billion dollars annually; according to recent data, roughly 1.7 billion small ruminants in Africa and Asia are at risk of PPR infection (Rahman, et al., 2020) (Vinayagamurthy, Naidu, & Roy, 2020) (Niedbalski, 2020). The relatively recent emergence of lineage IV PPRV in Africa and the Middle East and conversely the emergence of an African lineage in China increases the risk of its introduction into Europe (Vinayagamurthy, Naidu, & Roy, 2020). Since the launch of the Global Control and Eradication Strategy (GCES) of PPR by the FAO and OIE to rid the world of PPR by 2030, strides have been made in the raising of funds and increase in awareness campaigns (Jia , Wang, Liu, Meng, & Fan, 2020) (Herzog, et al., 2020). The GCES is a four stage process which includes assessment, control, eradication, and post eradication (Njeumi, Bailey, Soula, Diop, & Tekola, 2020). The development and use of recombinant vectored vaccines against PPR is crucial for the final stage of eradication. However, it is important to state that PPR vaccination response has been inconsistent due to influence by factors such as the environment and genetics therefore, it is necessary to have numerous vaccine trials to show the protective ability of potential vaccine candidates (Vinayagamurthy, Naidu, & Roy, 2020). Additionally, vaccination strategies need to go hand in hand with improved husbandry practices to lower risk of transmission in areas where

veterinary services are scarce (Herzog, et al., 2020). A major issue that has been addressed without mitigation strategies has been the presence of dead end hosts of PPR such as wild ruminant species (Furley, Taylor, & Obi, 1987) (Dou, et al., 2020) (Rahman, et al., 2020). This issue highlights the importance of continual vaccination against PPR after it has been eradicated in small ruminants to prevent cross species infections that may result in a new outbreak. While the current attenuated live vaccines against PPR are effective, their use will cease to exist in order to achieve disease free status (Niedbalski, 2020) (Njeumi, Bailey, Soula, Diop, & Tekola, 2020). To date, a vectored vaccine against PPR has not been used in the field, therefore commercialization and field use of vectored vaccines is of the utmost importance.

5.3 Significance and Shortcomings

CAPVs and PPRV are extremely important small ruminant and cattle diseases that cause significant damage in endemic countries from pastoral communities and small farms to the large scale import/export economy (Tuppurainen E. , et al., 2015) (Diallo, Bataille, Lancelot, & Libeau, 2019). Specifically, PPRV is considered the most important disease of small ruminants due to the severe effects it has on affected animals (Mariner, et al., 2016). Additionally, CAPVs have been attributed to be one of the main reasons in the hinderance of genetic improvement of small ruminants and cattle which would have otherwise allowed for the presence of large scale livestock production units in areas where they are endemic (Tuppurainen E. , et al., 2015). The rapid spread of LSDV into previously endemic free countries is an important reason to focus on the production of new types of vaccines to combat its spread (Mercier, et al., 2018). The use of CAPV DIVA vaccines would greatly aid in the effort to gain disease free status in endemic countries and to maintain that status in non-endemic countries. A DIVA vaccine has not been previously developed for any poxviruses therefore making the first DIVA vaccine would allow for exploration of a different and more efficient approach to current vaccination strategies and actively aid in the restoration of disease free status.

The major shortcoming of the research performed is the development of a DIVA companion test prior to the generation of a DIVA vaccine. The inability to remove the CP25 gene and inability to create a DIVA vaccine resulted in the DIVA companion test becoming a generic diagnostic blocking ELISA. This is not without merit however, because of the possible use of the companion test in performing routine diagnostic of CAPVs through the utilization of recombinant CP25. For future DIVA vaccine development, it is important to assess the likelihood DIVA vaccine generation before fully committing to developing a diagnostic test based on a particular protein.

6. FUTURE DIRECTIONS

6.1 CAPV DIVA Vaccines

New candidate genes which are both antigen and non-essential must be identified in order to develop a CAPV DIVA vaccine. The search is somewhat limited to proteins able to elicit a strong antibody response. From analysis of 25 total structural genes, there are 5 potential genes that may act as candidates, one of which may be ideal. The potential genes of interest are LSDV047, LSDV052, LSDV080, LSDV108, LSDV141. The first 4 genes are not in the ends of the genome which may imply a more essential role, however, due to lack of characterization their specific function as it applies to CAPVs has not been determined. Based on analysis of other poxviruses such as VACV, they are not conserved proteins. LSDV141 may be the most ideal candidate because of its proximity on the genome and lack of conservation. DIVA vaccine development needs to be initiated by the development of a serological assay, therefore, antigens from candidate genes need to be translated *in vitro* first. Once proteins are produced, they need to be analyzed for diagnostic utility as an indirect ELISA using positive and negative sera. Reactive proteins/protein would then be used to develop a blocking or competitive ELISA. Once an antigen is identified as a suitable diagnostic antigen, the next step of determining if the gene encoding the antigen is essential for virus replication. Constructs will be designed to remove the genes of interest from LSDV and SPPV vaccine strains. If these gene deleted viruses can be generated, then they can be evaluated as a DIVA vaccine with the companion diagnostic test.

6.2 Bivalent CAPV vaccine expressing PPR H

Animal experiments will be underway to evaluate the protective ability of the bivalent CAPV/PPRV vaccine. There will be groups of 5 sheep per experimental group. Sheep will be immunized intradermally with 10^4 TCID₅₀ vaccine then challenged with 2×10^6 TCID₅₀ virulent PPRV intranasally. Sheep will be monitored daily for temperature and clinical signs. Serum, oral swabs, and nasal swabs will be collected to quantify viral load and antibody response

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