Identification of cross-reactive epitope regions of bovine viral diarrhea virus and classical swine fever virus glycoproteins

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Mollie K. Burton

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Major Professor
Dr. Raymond R. R. Rowland

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Abstract

Pestiviruses such as classical swine fever virus (CSFV) and bovine viral diarrhea virus (BVDV) are some of the most economically important livestock diseases in the world. The antigenic similarities between members of the pestivirus genus allow for both BVDV and CSFV to infect swine. Infections with heterologous pestiviruses in swine can interfere with diagnostic tests for CSFV. The identification of cross-reactive and cross-neutralizing epitopes between CSFV and BVDV for the development of improved diagnostics and vaccines that allow for the differentiation of infected animals from vaccinated animals (DIVAs) are necessary to accurately detect and control CSFV. The overall goal of this research was to identify epitope regions recognized by antibodies that can differentiate between CSFV and BVDV. The approach was to use serum neutralization assays to confirm the presence of neutralizing antibodies to BVDV in swine serum collected from animals immunized with one of three separate Alphavirus vaccine constructs: BVDV-1b, CSFV E2, and CSFV E^{rns}. Results showed that animals immunized with the Alphavirus BVDV-1b construct had high neutralizing titers against BVDV-1a and animals immunized with Alphavirus CSFV E2 and E^{rns} constructs had low, but detectable, neutralizing activity. Polypeptide fragments of CSFV and BVDV E2 were then expressed in *E. coli* and purified using affinity chromatography. Serum from a pig immunized with the CSFV E2 Alphavirus construct was tested against two fragments of CSFV E2, 2/4 and 4/4, and four fragments BVDV E2, 1/4, 2/4, 3/4, and 4/4, using western blot analysis. Reactivity to fragments CSFV E2 2/4 and 4/4 and BVDV E2 1/4 and 4/4 was observed. The results of this study identified CSFV amino acid positions 774 through 857 and BVDV amino acid positions 783 through 872 as the regions that contain the epitopes recognized by cross-reactive antibodies

between BVDV and CSFV E2. These results provide more specific sequence regions to improve CSFV diagnostic assays and DIVA vaccines.

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Chapter 1 - Literature Review of Classical Swine Fever Virus and Bovine Viral Diarrhea Virus

Introduction

Pestiviruses such as classical swine fever virus (CSFV) and bovine viral diarrhea virus (BVDV) are some of the most economically important livestock diseases in the world. The antigenic similarities between members of the pestivirus genus allow for both BVDV and CSFV to infect swine. Infections with heterologous pestiviruses in swine can interfere with diagnostic tests for CSFV. For viruses that cause high morbidity and mortality like CSFV, outbreaks can be devastating and require efficacious vaccines coupled with quick and accurate diagnostics to control the virus and minimize the number of animals infected. Vaccines that allow for the differentiation of infected from vaccinated animals (DIVA) are increasingly needed for the control of pestiviruses. Without DIVA diagnostic tools it is difficult, if not impossible, to distinguish between an animal that has been naturally infected and one that has been vaccinated. Vaccines and accompanying diagnostic assays that have the necessary sensitivity and specificity to detect the pestivirus of interest are crucial for the control and surveillance of CSFV. In order to develop these types of assays and vaccines, unique areas or epitopes of each virus must be identified.

The *Flaviviridae* Family

The *Flaviviridae* family of viruses is a group of single-stranded, positive-sense RNA viruses that consists of four genera: *Flavivirus*, *Hepacivirus*, *Pestivirus*, and *Pegivirus*. Species such as yellow fever virus, West Nile virus, Japanese encephalitis virus, and dengue fever virus from the genus *Flavivirus* and hepatitis C virus (HCV) from the genus *Hepacivirus* are viruses

that cause severe disease in humans and are of major global concern (Lindenbach *et al.*, 2007; Stapleton *et al.*, 2011). Phylogenetic studies of the *Flaviviridae* family show that the *Pestivirus*, *Hepacivirus*, and *Pegivirus* genera are the most closely related. This close relationship between pestiviruses and hepaciviruses has been utilized to study viruses that replicate poorly in cell culture and lack available animal models. BVDV, a bovine pestivirus, has been used as a tissue culture surrogate for HCV as both viruses share similar genomic structure and replication strategies (Billerbeck *et al.*, 2013; Buckwold *et al.*, 2003; Ouzounov *et al.*, 2002).

The *Pestivirus* Genus

The genus *Pestivirus* consists of four main species of viruses: classical swine fever virus (CSFV), bovine viral diarrhea virus-1 (BVDV-1), bovine viral diarrhea virus-2 (BVDV-2), and border disease virus (BDV). Other unassigned species have been identified and include a giraffe pestivirus isolate, Giraffe-1 (Avalos-Ramirez *et al.*, 2001), HoBi-like viruses, also known as BVDV-3 or atypical pestiviruses (Schirrmeier *et al.*, 2004), pronghorn antelope pestivirus (Vilcek, 2001), and Bungowannah virus (Kirkland *et al.*, 2007). These viruses similarly infect a number of species in the order *Artiodactyla* such as domestic even-toed ungulates like cattle, sheep, and pigs as well as old- and new-world camelids and feral species such as deer, chamois, and antelopes. The worldwide geographic distribution of pestiviruses mirrors the large economic impact these viruses have on the livestock industry (Schweizer & Peterhans, 2014).

Virus

Economic Impact

CSFV, also known as hog cholera, swine fever, peste du porc, cólera porcina, and virusschweinepest, is a virus that solely infects members of the *Suidae* family (United States Department of Agriculture Animal and Plant Health Inspection Service [USDA APHIS], 2013).

CSFV is one of the most economically important diseases of swine worldwide due to the high morbidity and mortality of the disease. The virus, which was once widespread in the Americas, Asia, Africa, and Europe, has since been eradicated in a number of countries. The cost of infection can be devastating as seen in the 1997-1998 outbreak in the Netherlands which resulted in the destruction of approximately 1.1 million pigs and cost $2.3 billion to eradicate (Meuwissen *et al.*, 1999; Stegeman *et al.*, 2000). The first recorded case of CSFV in the United States dates back to 1833. The disease was officially eliminated from the country in 1978 after 16 years of eradication efforts (Edwards *et al.*, 2000; USDA APHIS, 2012). Current economic models of pork, swine, and related markets have estimated that a new outbreak in the United States would result in the loss of 11 million hogs and would cost $2.6-$4.1 billion (Paarlberg *et al.*, 2009). Today, CSFV remains endemic in a number of countries in Central and South America, Asia, Africa, and in wild boar populations of Europe (World Organization for Animal Health [OIE], 2009). The severity and highly contagious nature of CSFV has resulted in the listing of the virus by the United States Centers for Disease Control and Prevention (CDC) as a biosafety level 3 (BSL-3) agent *in vitro* and BSL-3-Ag *in vivo* (Wilson & Chosewood, 2009).

The Virion, Genomic Organization, and Proteins

CSFV is a single-stranded, positive-sense RNA virus (Meyers *et al.*, 1989; Moormann *et al.*, 1996, p. 199; Ruggli *et al.*, 1996). The CSFV capsid is hexagonally shaped with an electron-dense inner core structure of 30 nm surrounded by a spherical envelope 40-60 nm in diameter (Murphy *et al.*, 1995). The genome consists of a single open reading frame (ORF) flanked by a 5'-nontranslated region (NTR) and a 3'-NTR that encodes for a single polyprotein (Figure 1.1). The polyprotein is co- and post-translationally converted into four structural proteins: C, E^{rns}, E1, and E2 (Figure 1.2), and eight non-structural proteins: Npro, p7, NS2, NS3, NS4A, NS4B,

NS5A, and NS5B. The conversion of these proteins is performed by viral proteases Npro, NS2, NS3, and host cell proteases (Meyers & Thiel, 1996; Moormann *et al.*, 1990; Rümenapf *et al.*, 1993; Thiel *et al.*, 1991).

Glycoprotein E2, formerly E1 or gp51-55, is the major envelope protein expressed on the outer surface of the virion and contains four antigenic domains on the N-terminal half: A, B, C, and D with domain A further divided into subdomains A1-A3. E2 forms a heterodimer with E1, which is essential for virus entry, but is also found alone as a homodimer. This glycoprotein is not secreted as it has a hydrophobic transmembrane anchor at the C-terminus. The primary function of E2 is binding and entry of the virus into the host cell. It is the major determinant of cell culture tropism (Lindenbach *et al.*, 2007; van Rijn *et al.*, 1993, 1996; Ronecker *et al.*, 2008; Rümenapf *et al.*, 1993; Thiel *et al.*, 1991; Wensvoort, 1989).

The E^{rns} glycoprotein, formerly E2 or gp44/48, forms a homodimer in which the protein structure is stabilized by four intramolecular disulfide bridges. E^{rns} is atypical in that it lacks a transmembrane anchor and is anchored by an amphipathic helix formed by the folding of the C-terminus which allows for it to be secreted as a soluble protein from infected cells in addition to being associated with the mature virion. Functions of E^{rns} include ribonuclease activity, control of translocation across eukaryotic cell membranes, and it plays a role in inhibition of double-stranded RNA-induced cell responses. The N-terminus of E^{rns} is unique to pestiviruses, as compared to other members of the *Flavivirdae* family, and may be involved with the evasion of host interferon responses (Lindenbach *et al.*, 2007; Magkouras *et al.*, 2008; Meyer *et al.*, 2012; van Rijn *et al.*, 1996; Schneider *et al.*, 1993; Thiel *et al.*, 1991).

The phylogeny of CSFV is determined by the variable E2 fragment. To date there are three genotype groups each containing several subgroups. These genotypes consist of genotype 1

with subgroups 1.1, 1.2, 1.3, and 1.4; genotype 2 with subgroups 2.1, 2.2, and 2.3; and genotype 3 with subgroups 3.1, 3.2, 3.3, and 3.4 (Paton *et al.*, 2000, p. 200; Postel *et al.*, 2013). Each genotype is characteristic in certain geographical regions with groups one and two primarily in Europe and the Americas and group three circulating only in Asia (Moennig *et al.*, 2003; Paton *et al.*, 2000). Generally, genotype 1 and 3 isolates are of moderate or low virulence while genotype 1 isolates show the highest virulence. Some of the most widely used strains in research include highly virulent Brescia strain of genotype 1.2, highly virulent Alfort strain of genotype 1.1, and moderately virulent Paderborn of genotype 2.1 (Floegel-Niesmann *et al.*, 2003; Leifer *et al.*, 2011; Weesendorp *et al.*, 2009).

Host antibodies target the NS3, E2, and E^{rns} proteins; however, neutralizing antibodies are only produced towards E^{rns} and E2 glycoproteins. Moreover, only neutralizing antibodies against E2 have been shown to correlate with protective immunity in the host (König *et al.*, 1995; van Rijn *et al.*, 1992, 1993; Weiland *et al.*, 1992; Wensvoort, 1989). Further analysis of antibodies produced against glycoprotein E2 has shown that two structural units make up E2 with domains B/C and D/A forming two independent antigenic units. Both conformation-dependent and linear epitopes have been found on A and B/C domains with neutralizing antibodies produced against domains B/C; however, these domains are not conserved across CSFV strains. Analysis of the subdomains found that A1 and A2 are highly conserved across strains of CSFV, but only antibodies of subdomain A2 have been shown to be neutralizing (Chang *et al.*, 2010, 2012; Wensvoort, 1989; Wensvoort *et al.*, 1986, 1989a, 1990). Antibodies generated against NS3, due to the high genetic stability of the protein, are conserved across pestivirus species and are therefore not specific to CSFV alone (Beer *et al.*, 2007).

Virus Life Cycle and Replication

Attachment of the viral glycoproteins to the surface of host cells allows for the entry of CSFV. Glycoproteins E1 and E2 are essential for viral entry while E^{rns} is non-essential. Once the viral glycoproteins have bound to the host receptors, internalization occurs by clathrin-dependent endocytosis. The RNA viral genome is then released into the cytoplasm after the virus membrane fuses with the host endosomal membrane. Once inside the cell, RNA translation occurs in the cytoplasm. Replication is associated with cytoplasmic membranes and requires NS3, NS4A, NS4B, NS5A, and NS5B in addition to cellular components. The virion is then assembled and likely matures in intracellular vesicles which are then released by exocytosis (Lindenbach *et al.*, 2007).

Transmission

CSFV is present in blood and is secreted via most bodily discharges including urine, feces, and semen as well as oral, nasal, and conjunctival fluids. The natural and most efficient route of infection is through direct pig-to-pig contact and usually occurs oronasally. The consumption of swill containing products originating from CSFV-infected animals by susceptible pigs can also be a source of CSFV infection and has resulted in outbreaks in the past prompting bans on swill feeding in parts of Europe and in Australia (Dunne *et al.*, 1959; Edwards *et al.*, 2000; Moennig, 2000; Penrith *et al.*, 2011; Ribbens *et al.*, 2004). Artificial insemination can also be a source of infection, as the virus is excreted in semen, and stud boars should be screened for the virus prior to semen collection (Floegel *et al.*, 2000; de Smit *et al.*, 1999). The environment, people, and fomites, such as livestock trucks and tools used by veterinarians that have been contaminated with excretions of infected pigs, can also serve as sources of infectious material (Penrith *et al.*, 2011; Ribbens *et al.*, 2004, 2007).

Infection of a gestating sow during the first trimester will result in abortion of the piglets. Vertical transmission from sow to piglet can occur if the sow is infected in her second or third trimester of gestation. Some sows infected during the second trimester of gestation (days 50-70), during the development of the piglets' immune systems, can produce persistently infected (PI) animals via vertical transmission. Tolerance to the virus allows the virus to be shed throughout the animal's life with few or no clinical symptoms. The presence of PI animals within a herd or population can lead to the persistence of an outbreak even though they appear normal. Testing is required to identify such individuals as they are typically asymptomatic (Moennig, 2000; Moennig *et al.*, 2003; Ribbens *et al.*, 2004).

Wild boar also serve as important sources of CSFV, primarily in Europe, as the virus is endemic in many boar populations. Indirect contact is the most important route of transmission as virus can be spread through the feeding of silage that has been harvested from areas where wild boar have been or via contact with vehicles that have been used for transport of infected boar or carcasses. Direct contact with wild boar, while less likely, remains as a potential method of transmission to domestic pigs (Moennig, 2000; Penrith *et al.*, 2011; Ribbens *et al.*, 2004).

Other *Suiformes* such as the common warthog, bushpig, peccary/javelina, and pygmy hog are also susceptible to the virus (Barman *et al.*, 2012; Everett *et al.*, 2011; Fowler, 1996; Gers *et al.*, 2010). The epidemiological role of these species has not been fully explored and their role in transmission of the virus remains unknown (USDA APHIS, 2013). However, the possibility exists that persistently infected individuals of these species could become sources of infection for both domestic swine and other wild *Suiformes* (Barman *et al.*, 2012).

While the mechanisms are still not fully understood, it has been observed that the risk for secondary infections is highest in distance-dependent infections referred to as “neighborhood”

infections. The risk of these neighborhood infections increases as the distance to the primary infected herd decreases. It has been suggested that airborne transmission or undocumented biological or mechanical vectors may be the cause (Elbers *et al.*, 1999; Penrith *et al.*, 2011; Ribbens *et al.*, 2004). One study demonstrated that aerial transmission was possible in close quarters but that it was dependent on the virulence of the strain. Pigs infected with highly virulent strains, such as Brescia and Paderborn, excreted higher quantities of virus than those infected with the low-virulent Zoelen strain (Weesendorp *et al.*, 2008).

Infection and Immune Response

The immune response to CSFV is dependent on multiple factors including the virulence of the strain and the immunological status and age of the animal. Neutralizing antibodies can be detected as early as 3 days after infection while maximum antibody titers usually occur 3-4 weeks after infection and may remain elevated for as long as for 6 months (Chander *et al.*, 2014; Depner *et al.*, 1997; Laevens *et al.*, 1999; Moennig *et al.*, 2003; Moennig & Plagemann, 1992).

After the virus enters the host naturally through oral, nasal, conjunctival, and genital mucous membranes it infects the cells of the tonsil, the primary tissue for replication, and local oropharyngeal lymph nodes. CSFV has an affinity for cells of the mononuclear phagocyte system with the main targets consisting of macrophages, dendritic cells, and endothelial cells. The virus then infects secondary target organs such as the spleen, lymph nodes, thymus, bone marrow, and gut-associated lymphoid tissue (Knoetig *et al.*, 1999; Summerfield *et al.*, 1998, 2000; Summerfield & Ruggli, 2015; Susa *et al.*, 1992).

Generalized leukopenia is the major effect of CSFV infection with deficiency of B lymphocytes observed as a result of infection of the lymphoid organs. Lymphocyte apoptosis

induced by chemical mediators from monocyte-macrophage cells causes this immunosuppressive response (Chander *et al.*, 2014; Sánchez-Cordón *et al.*, 2002; Summerfield *et al.*, 2001). One hallmark of the acute disease phase, brought on by virulent strains of CSFV, is the extremely high levels of serum interferon-α (IFN-α), a signaling protein produced by leukocytes. These high levels of IFN-α coincide with depleted peripheral B and T lymphocytes. During the final stages of the disease, as much as 90% of T cell depletion has been shown to occur (Pauly *et al.*, 1998; Summerfield *et al.*, 2006; Summerfield & Ruggli, 2015).

Clinical Signs and Pathology

Signs of disease in animals infected with CSFV include fever, anorexia, dullness and apathy, conjunctivitis, and constipation with hard fecal pellets followed by diarrhea. Some animals may also display a staggering gait, ataxia, or convulsions. Purple skin discoloration or petechial hemorrhages may develop several days later on the ears, lower abdomen, and legs (Moennig *et al.*, 2003; Terpstra, 1991; OIE, 2014).

CSFV can occur in four forms: acute, chronic, subclinical, or persistently infected. Highly virulent strains of CSFV and infections in animals up to 12 weeks of age are associated with the acute form of the disease. Animals with the acute form of disease typically have an incubation period of 2-6 days and die 10-20 days after infection. CSFV strains of moderate to low virulence are associated with the subacute and chronic forms of the disease. Animals with the subacute form generally die 20-30 days after infection and those presenting with chronic infection will die much later after an apparent recovery followed by relapse. Persistently infected animals generally appear normal at birth and may develop normally for several weeks or months before clinical symptoms develop. Severe growth retardation and the development of runted pigs

are also associated with persistent infections (Blome *et al.*, 2006; Chander *et al.*, 2014; Dahle & Liess, 1992; Greiser-Wilke *et al.*, 2007; Moennig *et al.*, 2003; Terpstra, 1991; OIE, 2014).

Animals infected with high-virulent and moderately virulent strains of CSFV tend to show all of the symptoms previously described, while low-virulent strains may only present with poor reproductive performance of sows and piglets born with neurologic defects. Young animals tend to be more affected than older animals as they display classical hemorrhagic disease while older animals tend to experience a milder course of disease that may even be subclinical (Dahle & Liess, 1992; Greiser-Wilke *et al.*, 2007; Moennig *et al.*, 2003; Terpstra, 1991; OIE, 2014).

In subclinical cases no gross changes are found upon necropsy. In cases of acute or subacute infection hemorrhages can be found in various organs throughout the body. The lymph nodes generally appear swollen, edematous, and are red or black in color due to diffuse hemorrhages. Hemorrhages ranging in size from petechial to echhymotic can be present on the skin, heart, urinary bladder, larynx, intestinal mucosa, and serosa. The skin may also be cyanotic and the kidneys will show petechiae in the cortex, also known as having a “turkey egg” appearance. Splenic infarcts up to 10 mm in size are considered to be characteristic of acute infections. In the large intestine, the presence of button ulcers and the general appearance of being hyperemic are characteristic in subacute and chronic infection. Brain and spinal cord lesions are known to occur along with congestion in the lungs, liver, and bone marrow. Atrophy of the thymus and depletion of lymphocytes in tonsils, lymph nodes, and spleen are also observed (Blome *et al.*, 2006; Chander *et al.*, 2014; Terpstra, 1991; OIE, 2014).

The symptoms of CSFV can be difficult to differentiate from those caused by other hemorrhagic and febrile diseases of swine, such as African swine fever virus (ASFV), porcine dermatitis and nephropathy syndrome (PDNS), porcine reproductive and respiratory syndrome

virus (PRRSV), post-weaning multisystemic wasting syndrome (PMWS), bacterial septicemias, anticoagulant poisoning, and hemolytic disease of the newborn, and require differential testing to confirm which disease is affecting the animal. Septicemic diseases, such as erysipelas, salmonellosis, and *Haemophilus suis* infections, must also be considered as differential diagnoses (Floegel-Niesmann *et al.*, 2003; Greiser-Wilke *et al.*, 2007; Moennig *et al.*, 2003). Infection with a bovine pestivirus, BVDV, can also present with symptoms that resemble CSFV infection (Terpstra & Wensvoort, 1988).

Detection of CSFV

Diagnostic methods for CSFV include reverse transcription-polymerase chain reaction (RT-PCR), fluorescent antibody tests (FAT), virus isolation, enzyme-linked immunosorbent assays (ELISA), fluorescent antibody virus neutralization tests (VNT), neutralizing peroxidase-linked assays (NPLA), and immunoperoxidase staining using monoclonal antibodies for differentiation of pestiviruses (Blome *et al.*, 2006). For diagnosis in live animals, the detection of virus or viral nucleic acid in blood or antibodies in serum are the methods of choice (OIE, 2014). However, it takes two to three weeks post infection for antibodies to develop (Greiser-Wilke *et al.*, 2007). As a result, antibody detection methods do not provide an accurate diagnosis during early infection. While ELISAs and VNTs are the recommended methods for detection of an immune response to CSFV, cross-reactive antibodies against other pestivirus species have been observed in pigs. Therefore, screening tests should be followed by confirmatory tests that are specific for CSFV. The method of choice is the comparative neutralization test which compares neutralizing titers of antibodies to those of other pestivirus species. For diagnosis in deceased animals, detection of virus, viral nucleic acid, or antigen in organ samples through virus

isolation, direct FAT, or RT-PCR are suitable methods (Carbrey *et al.*, 1969; Fernelius *et al.*, 1973; OIE, 2014).

Control and Prevention

Vaccination, culling of infected animals or those within contact range (stamping out), disinfection of infected areas, quarantine, movement control, precautions at borders, screening, surveillance, and control of wildlife reservoirs are all commonly implemented methods of control (OIE, 2015a). While the safety and efficacy of vaccines for CSFV has been shown with no evidence of reversion, a number of countries where the virus is endemic, such as those in the European Union, have banned vaccination due to the inability to differentiate antibodies produced by vaccinated animals from those naturally infected with the virus (Greiser-Wilke & Moennig, 2004; USDA APHIS, 2012). This ban on vaccinations in the European Union is due to the fear that during an outbreak vaccinated pigs may become apparently healthy carriers of the virus and further spread the disease (Council of the European Union, 2001).

Vaccines for CSFV include a number of live attenuated vaccines (LAV)/modified live virus (MLV) vaccines. The most commonly used attenuated vaccines include the Chinese lapinised strain (CLS or C-stain), the Japanese guinea pig cell-culture-adapted (GPE-) strain, the Thiveral strain, and the Mexican PAV strain. The C-strain vaccine is the most widely used as it provides protection against all genotypes and is effective three days post vaccination. LAVs can also be administered orally and have been used to vaccinate wild boar in Europe (Beer *et al.*, 2007; Graham *et al.*, 2012; Greiser-Wilke & Moennig, 2004; Huang *et al.*, 2014).

DIVA vaccines, also known as marker or subunit vaccines, are the focus of current vaccine development efforts and few are commercially available. A number of these vaccines utilize the E2 or E^{rns} glycoprotein to induce a neutralizing antibody response and use companion

ELISAs to determine the presence or absence of the glycoproteins. For example, animals naturally infected with CSFV will produce antibodies against both E2 and E^{rns}. Through DIVA vaccination, animals will only develop an antibody response against the glycoprotein they are vaccinated with and lack antibodies against the other glycoprotein, and are therefore negative on the companion ELISA (Beer *et al.*, 2007).

Chimeric pestiviruses are one type of DIVA vaccines that are currently under development. The most promising of these, CP7_E2alf, was produced by replacing the E2 gene of BVDV strain CP7 with the E2 gene of CSFV strain Alfort 187. These modified live chimeric vaccines have comparable protection to that of the Chinese strain vaccines, can be administered orally, and utilize a companion E^{rns} ELISA (Beer *et al.*, 2007; Huang *et al.*, 2014; Rasmussen *et al.*, 2007; Reimann *et al.*, 2004, 2016; USDA APHIS, 2012). Another DIVA chimeric pestivirus that has also been under development is CP7_E2gif. This vaccine also uses a BVDV strain CP7 backbone in which the E2 gene is replaced by that of BDV Gifhorn E2 and utilizes a companion E2 ELISA (Rasmussen *et al.*, 2007; von Rosen *et al.*, 2014). Other types of DIVA vaccines also under development include CSFV peptide vaccines, DNA vaccines, and viral vector vaccines. (Beer *et al.*, 2007).

Bovine Viral Diarrhea Virus

Economic Impact

First identified in 1957, BVDV is a virus that infects cattle and other ruminants. BVDV can be found worldwide in cattle populations with 60-85% of cattle testing seropositive. Economic losses due to BVDV infection in the United States have been estimated at $20 million per million calvings for low-virulent strains of the virus and as high as $57 million per million calvings for high-virulent strains (Houe, 1999). BVDV eradication efforts are ongoing in a

number of European countries such as Switzerland, Norway, Germany, Ireland, and Scotland (Barrett *et al.*, 2011). In Switzerland, using data collected in 1995-1997, it was estimated that BVDV induced losses were around 9 million Swiss francs per year (Institut für Veterinär-Virologie, 2006). The Swiss BVD-eradication program, which began in 2008, is expected to cost approximately 40 million euros with a third of this amount being paid for by farmers (Presi *et al.*, 2011; Presi & Heim, 2010). BVDV, due to its infectious nature, is categorized as a BSL-2 agent by the CDC (Wilson & Chosewood, 2009).

The Virion, Genomic Organization, and Proteins

BVDV is a positive-sense, single-stranded nonpolyadenylated RNA virus about 12.5 kb in size with one large ORF flanked by 5' and 3' NTRs. The virion is enveloped and spherical in shape with a diameter of 40-60 nm. The genome organization and viral protein cleavage products are the same as those of CSFV (Figure 1.1) (Lindenbach *et al.*, 2007; Meyers & Thiel, 1996). The virus life cycle and replication method are also the same as what was described previously for CSFV (Lindenbach *et al.*, 2007).

Structural glycoprotein E2, formerly gp53, and glycoprotein E^{rns}, formerly gp48, share the same features and functions as those of CSFV (Deregt *et al.*, 1998; Lindenbach *et al.*, 2007; Paton *et al.*, 1992). The current literature disagrees on the antigenic domains of BVDV E2 as one study identified three antigenic domains, I-III, and other identified four domains, DA, DB, DC, and DD (El Omari *et al.*, 2013; Li *et al.*, 2013; Wang *et al.*, 2015). Domain I was identified between amino acid positons 693-782, domain II between amino acids 783-860, and domain III between amino acids 861-1035 (Li *et al.*, 2013). As with CSFV, host antibodies are made against NS3, E2, and E^{rns} proteins with E2 being the most immunodominant (Corapi *et al.*, 1990; Deregt *et al.*, 1998; Lindenbach *et al.*, 2007; Paton *et al.*, 1992).

There are two genotypes of BVDV: BVDV-1 and BVDV-2. The two genotypes have only 60% homology between the nucleotide and amino acid sequences of E2 and 75% homology between the 5' NTR (Donis, 1995; van Rijn *et al.*, 1997; TIJSSEN *et al.*, 1996). Analysis of the 5' NTR and Npro regions have shown the presence of 20 subgenotypes of BVDV-1 which include BVDV-1a through BVDV-1t (Giammarioli *et al.*, 2014) and six subgenotypes of BVDV-2 which include BVDV-2a through BVDV-2f (Giangaspero & Harasawa, 2004). The main classical strains of BVDV, such as NADL, SD-1, Oregon, Singer, Osloss, and NY1 are all of BVDV-1a or BVDV-1b subgenotypes. Different subgenotypes tend to be predominant in different geographic regions. BVDV-1a is widely distributed in the United States and Canada and predominates in the UK while BVDV-1b is prevalent in continental Europe and the United States. While prevalent in the United States and continental Europe, genetic typing showed that BVDV-2 is less prevalent than BVDV-1 in Europe (Kalaycioglu, 2007).

Two biotypes of the virus exist, cytopathogenic (cp) and non-cytopathogenic (ncp), which are based on their effects on cells *in vitro*. Cytopathic biotypes will induce apoptosis in cultured cells while non-cytopathogenic biotypes do not. Acute infections are generally caused by noncytopathogenic biotypes, although cytopathogenic biotypes have been shown to induce acute infection under experimental conditions (Lanyon *et al.*, 2014).

Transmission and Clinical Signs

BVDV can infect its host two ways: transiently or persistently. Seronegative animals that become infected and shed virus are said to be transiently infected. However, the majority of transiently infected animals will have no manifestation of any clinical signs (Ames, 1986). Those cattle that become infected and display clinical signs of disease are said to have acute BVDV infection. Acute infections are typically characterized by clinical signs such as fever,

anorexia, lethargy, leukopenia, ocular and nasal discharge, oral lesions, diarrhea, reduced fertility, abortion, stillbirths, congenital defects, and decreased milk production. The acute presentation of BVDV has an incubation period of 5-7 days and viremia can last up to 15 days. Generally, the time in which cattle with acute BVDV infections recover is related to duration of viremia and severity of lesions, both of which are associated with virulence of the strain, in addition to the presence of any secondary infections. However, most animals recover within 2-4 weeks after the onset of clinical signs (Evermann & Barrington, 2005).

Calves that are exposed to noncytopathogenic strains of BVDV between 30-125 days of gestation during which time the immune system is developing will become PI animals. The immune systems of these animals develop tolerance to the virus. As a result, the animals do not mount an immune response the virus and are born as animals that are persistently viremic. Most PI calves are characterized as "poor-doers" that survive only a few a few hours or days. Longer surviving animals generally appear normal at birth but have stunted growth. These animals are also 50% more likely to die within the first year of life (Liebler-Tenorio, 2005). PI calves can and do survive into adulthood, although both cows and bulls will have reduced reproductive performance. Cows are able to conceive and give birth, however they will always give birth to PI calves (Grooms *et al.*, 1996; Liebler-Tenorio, 2005).

The appearance of a fatal condition known as mucosal disease can arise in PI cattle as the result of infection with cytopathic BVDV through recombination between noncytopathogenic biotypes, superinfection, or mutation of the persistent biotype (OIE, 2015b). Mucosal disease presents in both acute and chronic manifestations. Symptoms of acute mucosal disease may last from 3-10 days before death occurs and can include fever, anorexia, polypnea, and tachycardia; profuse, watery diarrhea; erosions on the tongue, palate, and gingiva; lacrimation, excessive

salivation, and ocular and nasal discharge; ocular edema and inflammation of the interdigital space and coronary bands (Bolin, 1995; Evermann & Barrington, 2005). In some cases death can occur so suddenly that it may be the only clinical sign (Brownlie *et al.*, 1987). Mucosal disease epizootics have been known to occur. These outbreaks occur when groups of cows are bred at the same time and subsequently become infected with BVDV around the same time of gestation. Ultimately this results in all of them producing PI calves. When cytopathic BVDV infects one PI animal it often infects all of the PI animals resulting in a sudden onset of animals with mucosal disease. Some animals with mucosal disease develop a chronic form of the disease that presents as unthrifty appearing animals with persistent loose feces or intermittent diarrhea, mild-to-moderate anorexia, chronic recurrent bloat, interdigital erosions, nonhealing erosive skin lesions, ocular and nasal discharge, alopecia and hyperkeratinization around the head and neck, chronic laminitis, and abnormal hoof growth. Animals with the chronic form of mucosal disease rarely survive longer than 18 months of age (Evermann & Barrington, 2005).

Noncytopathogenic and cytopathogenic biotypes of BVDV can be transmitted in bodily fluids such as nasal discharge, urine, milk, semen, saliva, tears, and fetal fluids. During acute infection, viral shedding occurs for about 10 days following infection with detectable antibodies occurring 13-19 days after exposure. Animals with primary BVDV infection have a relatively short period of viral shedding compared to PI animals and are generally poor transmitters of the virus. Direct contact with an infected animal or fomites are the most likely sources of infection, but transmission through ambient air has been documented (Niskanen & Lindberg, 2003; OIE, 2015b). Secretions and contact with PI calves are the most plausible and effective route for transmission of BVDV and can perpetuate infection within a herd (Niskanen & Lindberg, 2003). PI cattle are also the most important source of noncytopathogenic BVDV, as they shed the virus

continuously throughout their lives and can be asymptomatic (Lindberg & Alenius, 1999). One study showed that PI calves are likely the most important reservoir of BVDV for susceptible cattle with 70-100% of susceptible penmates becoming infected after exposure to PI animals (Fulton *et al.*, 2009).

PI breeding bulls are a major concern as a means of viral transmission through infected semen. PI bulls can be a significant source of virus as the amount of BVDV excreted in the semen is extremely high, as compared to transiently infected bulls, and the semen can be quickly dispersed anywhere in the world. Another animal product that can serve as potential route of transmission includes embryo transfer materials, as the virus can be present in the collection fluid or on the zona pellucida and can be horizontally transferred to the recipient cow (Larson *et al.*, 2004). Contaminated laboratory supplies used in vaccines are also potential sources of BVDV infection and should be checked for BVDV status by the vaccine manufacturer prior to use or be sourced from a supplier that tests its stock (Fulton, 2015).

Other species, such as sheep, have been documented to transmit BVDV to cattle. While species such as swine can be infected with BVDV, there is currently no evidence of transmission to cattle (Larson *et al.*, 2004). Seroprevalence studies looking for seroconversion of BVDV in wild ungulate populations found that pronghorn, caribou, deer, elk, moose, reindeer, and bison were antibody positive as well as domestic ungulates such as alpaca and llamas (Aguirre *et al.*, 2014; Duncan *et al.*, 2008; Larska, 2015). One study identified a mousedeer as a PI animal infected with BVDV-1f. Examination of the virus revealed only a few nucleotide changes when compared to the strain obtained from German cattle. While wild mousedeer represent a low risk of infecting domestic cattle, the isolation of the virus shows the wide range of ungulates that can be infected and potentially spread the virus (Uttenthal *et al.*, 2005).

Detection of BVDV

Diagnostic methods for BVDV include virus isolation, ELISA, immunohistochemistry, RT-PCR, VNTs, and virus isolation in cell culture. Diagnostic testing has been focused on detecting PI animals as a means of viral control (Fulton *et al.*, 2009; World Organization for Animal Health, 2008). BVDV antigens from PI calves can be detected in skin biopsies by immunohistochemical staining. However, maternal antibodies can impact testing methods in calves because BVDV maternal antibodies may block viral infectivity or detection of viral antigens in these animals. Moreover, maternal antibodies may be detected in PI animals for up to three months and are detectable in non-PI calves for up to eight months (Sandvik, 2004).

When surveying for BVDV using ELISAs, it is important to consider which viral antigens are used as BVDV-1 and BVDV-2 are two separate species. For this reason, and for optimal sensitivity, it is recommended that ELISAs detecting NS2 and NS3 be used over those utilizing E^{rns} (Sandvik, 2005).

Control and Prevention

Surveillance programs to identity PI animals for removal remains the best way to control the virus. Biosecurity measures, vaccination, and the removal or isolation of PI animals are all part of successful BVDV control programs (Fulton, 2015). Self-clearance of the virus from smaller BVDV-positive herds with no intervention has been documented to occur. This results from a lack of susceptible animals, as animals seroconvert and develop immunity to the virus, in addition to the removal of any PI animals. For surveillance of the virus in PI animals and those acutely infected, diagnostic assays such as BVDV antibody and antigen ELISAs are commercially available. ELISAs for the testing of bulk milk samples are also available commercially as a means for prevalence surveillance in dairy herds (Sandvik, 2004).

While surveillance programs targeted at identifying and removing PI animals are the most effective way to prevent subsequent BVDV infections in herds, vaccination remains an important tool for cattle producers in areas where high levels of beef production occur and where the virus is endemic. There are a number of MLV and inactivated (killed) BVDV vaccines that are commercially available. The MLV vaccines are believed to induce a more rapid immune response as they only require a single dose while killed vaccines require a booster. Vaccines for BVDV should include both BVDV-1 and BVDV-2 to provide the best protection against the virus. The majority of vaccines, both MLV and killed, use BVDV-1 cytopathogenic strains such as Singer, NADL, and C24 and a cytopathogenic strain of BVDV-2 (Fulton, 2015).

Vaccines that are currently available lack DIVA properties and make surveillance for BVDV more complicated in areas where animals have been vaccinated against the virus. Several BVDV DIVA vaccines are still in the experimental phase. Of these, the Alphavirus-derived replicon particle system is one of the most effective as the antibodies produced have been shown to cross-neutralize both BVDV-1 and BVDV-2 following booster vaccination (Loy *et al.*, 2013). Alphaviruses are a group of single-stranded RNA viruses and belong to the family *Togaviridae* and include viruses such as Semliki Forest virus (SFV), Venezuelan equine encephalitis (VEE) virus, and Sindbis virus (SIN). For the development of vaccines against infectious diseases, like BVDV, the virus is genetically modified to express substituted foreign genes of interest. The resulting replicon and helper vector supplying the viral structural genes are then co-transfected into a mammalian cell line via electroporation. Once in the cells the replicon and helper components multiply and form many new alphavaccine particles which contain the antigen of interest. After 24 hours, the particles are harvested from the cells and are purified and formulated. The resulting vaccine construct is a single-cycle replication-deficient recombinant

virus-like replicon particle that will only express the substituted genes (‘AlphaVax’, 2016; Lundstrom, 2014). Another experimental DIVA vaccine is a pronghorn antelope pestivirus/BVDV chimera. To generate this chimeric virus, a BVDV backbone was utilized and the E^{rns} sequence of the BVDV backbone was replaced by that of the pronghorn antelope pestivirus. This chimera was distinguishable from BVDV infection by anti-E^{rns} serology after infection and is a potential DIVA vaccine candidate (Luo *et al.*, 2012; Mogler & Kamrud, 2014).

The Pestivirus Problem

The immunological and genetic similarities between pestivirus species allows for BVDV to infect swine. During the CSFV outbreak in the Netherlands in 1997-1998, serum that tested positive on ELISA for CSFV was further characterized by VNT. The results of the VNTs showed that only 15% of the samples were truly positive for CSFV while 35% were positive for BVD/BVDV. Serum that tested positive for ruminant pestivirus neutralizing antibodies most often originated from sows and the prevalence varied from 0 to 60% (de Smit *et al.*, 2000). Current surveys of swine have shown the seroconversion rate to be as high 64% in China and anywhere from 2-42% in North American swine herds. Sources of BVDV infection are believed to be cattle in close proximity to swine herds. However, during the 1997-1998 outbreak, a majority of the animals that tested positive for BDV/BVDV were not kept in close proximity to ruminants (de Smit *et al.*, 2000; Tao *et al.*, 2013). Other possible sources of infection for pigs include the feeding of BVDV contaminated milk and cow offal as well as contaminated swine vaccines. In CSFV vaccines, the contamination rate has been shown to be as high as 21.74% due to inefficient screening procedures for BVDV and contaminated bovine serum used in the manufacturing of these vaccines (Tao *et al.*, 2013; Wensvoort & Terpstra, 1988).

BVDV infection in pigs has been shown to cause all of the clinical signs of CSFV, such as high fever, lethargy, yellow diarrhea, and skin lesions of the ears, abdomen, and legs. While BVDV infection in pigs can be symptomatic, it usually occurs without clinical signs which allows the virus to spread undetected. In addition to the typical symptoms of CSFV infection, BVDV infected pigs have been shown to have breeding problems with aborted, stillborn, and malformed piglets occurring (Carbrey *et al.*, 1976; Castrucci *et al.*, 1974; Tao *et al.*, 2013; Terpstra & Wensvoort, 1988, 1997; Walz *et al.*, 1999).

Current data suggest that both CSFV and BVDV share the same receptor for entry into host cells. Bovine cluster of differentiation 46 (CD46) was first identified as the cellular receptor for BVDV and porcine CD46 was later identified for CSFV with further analysis showing roughly 55% sequence identity at the amino acid level between BVDV and CSFV CD46. CD46, also known as membrane cofactor protein, is present on all nucleated cells and belongs to the family of regulators of complement activation. Specifically, CD46 functions as a cofactor for plasma serine protease factor which cleaves complement factors C3b and C4b that have deposited on host tissues therefore preventing further complement activation (Dräger *et al.*, 2015; Krey *et al.*, 2006a; Maurer *et al.*, 2004).

Other cells expressing CD46, such as HeLa and mouse L cells, have been shown to absorb BVDV into the cell. However, no productive infection occurred from these experiments suggesting that BVDV interacts with one or more additional cellular molecules that facilitate viral entry. This data would also suggest that these other cellular molecules are coreceptors and are likely present on cells of species belonging to the order Artiodactyla (Maurer *et al.*, 2004).

Several other receptors have been identified to be involved with viral binding and support the theory previously stated. Low density lipoprotein (LDL) receptor was first identified as a

common receptor for a number of *Flaviviridae* viruses, including HCV and BVDV, and mediates endocytosis of the virus. However, anti-LDL antibodies were shown to only delay the cytopathic effects of BVDV on cells while complete inhibition was observed for HCV, further supporting the theory of multiple receptors for pestivirus entry into the host cell (Agnello *et al.*, 1999; Krey *et al.*, 2006b). Another receptor that was identified is heparan sulfate (HS) which was shown to be a cellular receptor for both viruses and interacts with E^{rns}. However, binding of the virus to HS alone is also not sufficient for viral entry into the host cell (Dräger *et al.*, 2015; Hulst *et al.*, 2001). The laminin receptor (LamR) has also been shown to be responsible for viral attachment to the host cell in CSFV infection and specifically interacts with E^{rns} (Chen *et al.*, 2015). Annexin 2 (Anx2) was also demonstrated to function as a receptor for CSFV and is associated with E2 (Yang *et al.*, 2015).

Analysis of the crystal structure of BVDV E2 further supports the similarities between CSFV and BVDV E2. As mentioned previously, CSFV E2 has four antigenic domains, A-D, and BVDV E2 has three, I-III. Domain II of BVDV E2 was mapped to domains A/D of CSFV E2 and domains B/C correspond to domain I of BVDV E2. It is suggested that domain III of BVDV E2 does not contain any antibody epitopes because it is not exposed to the viral surface which is likely a result of the C-terminal hydrophobic transmembrane anchor (Li *et al.*, 2013).

The genetic characteristics of BVDV strains in pigs remains unknown for the swine populations of Europe and America. In three viral genomic sequences obtained from isolates in Chinese pigs, all three were noncytopathogenic with two identified as BVDV-1 and one BVDV-2. Between these BVDV-1 and -2 strains, only 70% homology was shown between the two (Tao *et al.*, 2013).

Epitope Mapping

Epitope mapping is the process of identifying the interaction site on an antigen where the antibody binds. Epitope mapping is essential for the development of new diagnostic assays and vaccines. Once identified, these epitopes can be used to generate more precise targets for diagnostic assays and subunit vaccines. Epitope-based vaccines are desirable over MLVs because they are unable to revert to a virulent form. These vaccines generate an optimal immune response with no risk of reversion as they contain just the essential antigens to generate an immune response (Gershoni *et al.*, 2007; National Institutes of Health National Institute of Allergy and Infectious Diseases, 2012).

The epitope of interest can change depending on which part of the adaptive immune response is the desired target. T cell epitopes differ from B cell and antibody epitopes in that T cell epitopes result from antigen processing by professional antigen presenting cells (APCs). The resulting peptide is about 10 amino acids in length and is presented in major histocompatibility complex (MHC) type II on the surface of the APC; however, because of the open-ended binding groove on MHCII, this length can vary with differing numbers of amino acids that extend out of the binding groove. The epitopes presented in MHC II are linear and are not conformational due to processing of the peptide by the APC as it breaks down the tertiary structure of the protein. B cell receptors (BCR) and antibody paratope binding regions consist of many overlapping groups of 15 amino acids that are approximately 50 variable amino acids in length. As a result, BCRs and antibodies have a large number of potential paratopes. Unlike T cells, BCRs and antibodies are not limited to antigen peptides presented in MHC II and can bind antigen peptides directly. As antigens can vary in shape and size, the epitope to which a BCR or an antibody can bind can be continuous (linear) or discontinuous (conformational),

depending on where the antibody may bind (Frank, 2002; Owen *et al.*, 2013; Pandit *et al.*, 2012). Linear epitopes are formed by several adjacent amino acids and are generally about six amino acids in length. Large antigens with tertiary and/or quaternary structures, such as proteins in their native conformation, generally have epitopes that are formed by amino acids that are not in sequence but are spatially juxtaposed in the folded protein in what are known as conformational epitopes. However, the denaturing of a protein, or loss of the primary, secondary, tertiary, and quaternary structures, will result in a linear chain of amino acid residues. In these cases, the conformational epitopes are no longer available for antibodies to bind, only the linear epitopes (Abul K. Abbas, 2005).

Common methods of epitope mapping include X-ray co-crystallography, peptide scanning (also called overlapping peptide scanning or pepscan analysis), phage display library scanning, site-directed mutagenesis and mutagenesis mapping, and hydrogen/deuterium exchange mass spectrometry. X-ray co-crystallography is currently considered the gold standard as this method generates an atomic resolution of the epitope (Gershoni *et al.*, 2007; Ossipow & Fischer, 2014).

Cross-protection of Antibodies

Cross-protective immunity, or cross-reactivity, occurs when antibodies specific for one antigen or epitope from one organism are also protective against a similar but different species of organism. Due to the similarities of the different pestivirus species, cross-reactive and cross-neutralizing antibodies have been documented. The issue arises when animals are tested for one pestivirus species and have previously been infected and seroconverted, or produce detectable antibodies in the blood, to a different pestivirus species. The presence of these similar antibodies may cause a positive test result for a disease when the animal was not infected with the disease

of interest, also known as a false positive. False positives can also occur due to contaminated laboratory supplies, such as fetal bovine serum used to manufacture vaccines (Carbrey *et al.*, 1969; Edwards *et al.*, 1991; Makoschey *et al.*, 2003; Wensvoort *et al.*, 1989b).

Previous studies have assessed the cross-protective immunity of BVDV antibodies to CSFV. In one study, pigs were first infected with BVDV type 1b strain St. Oedenrode, isolated from a naturally infected pig, and later challenged with the CSFV strain Paderborn, a moderately virulent strain. The results of the study showed that the presence of BVDV antibodies can protect against the clinical signs of a CSFV infection and limit transmission of CSFV. It was noted that diagnostic assays, such as the NPLA, while able to rule out CSFV in BVDV infected animals, may be unable to detect a current CSFV infection in animals that have BVDV antibodies (Wieringa-Jelsma *et al.*, 2006).

Another study with the aim of evaluating current CSFV antibody detection assays found that only one out of the seven tests that were analyzed, using samples of BVDV-1, BVDV-2, and BDV, was unable to differentiate between CSFV and the other pestiviruses. The other assays that were examined were negative anywhere from 44% to 86% of the time. The assays for E2 had the best results while those using NS3 and E^{rns} were less accurate (Schroeder *et al.*, 2012).

Purpose

The purpose of this study was to determine the epitope regions of cross-reactivity between CSFV and BVDV glycoproteins as a means to identify epitopes that differentiate between the two pestivirus species. The worldwide distribution of BVDV coupled with the virus's ability to infect swine makes the detection and control of CSFV more difficult. Outbreaks of CSFV can be devastating and require efficacious vaccines coupled with quick and accurate diagnostics to control the virus and minimize the number of animals infected. Diagnostic assays

with improved specificity and DIVA marker vaccines are essential for the surveillance of and protection against this economically important virus. The identification of these unique regions is necessary for the development of improved CSFV diagnostic assays and DIVA vaccines.

Tables and Figures

Figure 1.1 Genomic Organization of CSFV and BVDV

The genomic organization of both CSFV and BVDV. The genome consists of a single open reading frame (ORF) flanked by a 5'-nontranslated region (NTR) and a 3'-NTR that encodes for a single polyprotein that is converted into four structural proteins: C, E^{rns}, E1, and E2, and eight non-structural proteins: Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.

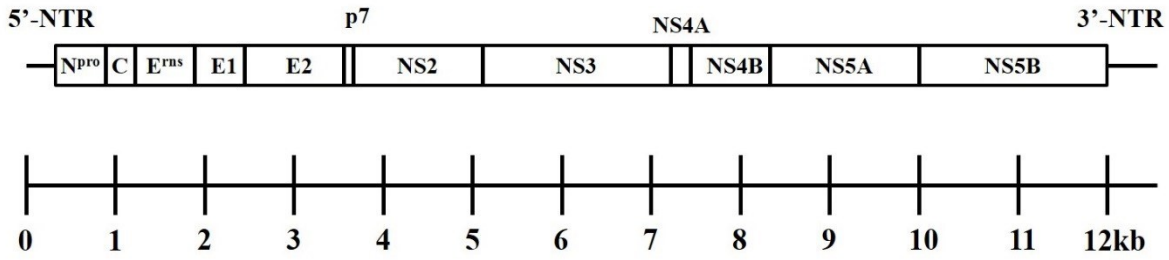
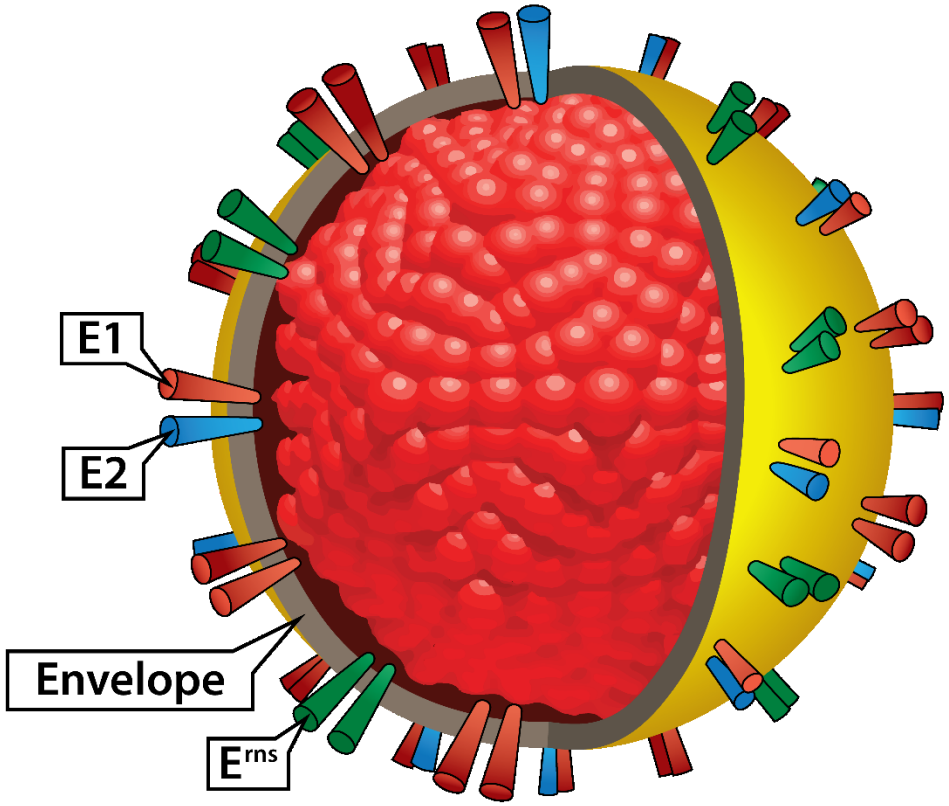


Figure 1.2 Structural Organization of CSFV and BVDV

Glycoproteins E1, E2, and E^{rns} are expressed on the outside of the virus forming E1 homodimers, E1/E2 heterodimers, and E^{rns} homodimers. E1 and E2 are both anchored into the viral envelope by a transmembrane region and E^{rns} is anchored by an amphipathic helix formed by the folding of the C-terminus.



Chapter 2 - Materials and Methods

Experimental Serum Samples

The 13 serum samples and one control serum sample used for the serum neutralization assays were collected from a previous study in which 3- to 4-week-old weanling Large White and Landrace crossbred domestic pigs were immunized with alphavirus-based vaccine constructs. The viral vaccine constructs were created by cloning genes for CSFV E2, CSFV E^{rns}, BVDV-1b E2, or ASFV HAp72 separately into a Venezuelan equine encephalitis (VEE) vector. The VEE virus expressing the gene of interest was grown in Vero cells and the culture fluid was used to prepare the vaccines as previously described (Bosworth *et al.*, 2010). After acclimation, animals were vaccinated separately with one of the viral vector constructs and subsequently received booster immunizations at days 21 and 42 post vaccination. The serum used for this project was collected on day 57 post vaccination.

Maintenance of Cells

Bovine turbinate (BT) cells (Kansas State University Veterinary Diagnostic Laboratory) were maintained in T75 cell culture flasks with Eagle's Minimum Essential Medium (MEM) with Earle's salts and sodium bicarbonate and without L-glutamine (Sigma-Aldrich) to which fetal bovine serum (FBS) (Sigma-Aldrich), Fungizone®, active ingredient amphotericin B, (Life Technologies), Pen Strep (penicillin and streptomycin) (Life Technologies), and 100X concentrate GlutaMAX™-1 (Thermo Fisher Scientific) had been added. The final concentrations of these additives were as follows: FBS 72 ml/L, amphotericin B 3 mg/L, penicillin 80,000 U/L, streptomycin 80 mg/L, and 100X GlutaMAX™ 10 ml/L. Cells were incubated at 37 °C and

5% CO_2. Using sterile technique, cells were passaged every 3-5 days once they had reached approximately 80% confluency.

Virus Propagation

BVDV-1a Singer strain stock virus (Kansas State University Veterinary Diagnostic Laboratory) was propagated via the addition of 400 µl of virus with a 50% tissue culture infectious dose ($TCID_{50}$) of approximately 1 x 10^6/ml to a T75 flask of approximately 40% confluent BT cells. The flask was incubated for 6 days at 37 °C and 5% CO_2. In order to lyse the cells, the flask was placed at -80 °C until frozen. The flask was subsequently thawed at 37 °C and the media was aliquoted into 1.5-ml cryovials and stored at -80 °C.

Calculation of $TCID_{50}$

End-point titration assays were performed to calculate the $TCID_{50}$ of the propagated stock virus. Prior to performing the assays, BT cells were seeded in 96-well tissue culture plates until approximately 80% confluent. Stock virus was serially diluted 1:10 in maintenance media and 200 µl of each dilution was added in quadruplicate to the 96-well tissue culture plates of BT cells. The cells were incubated at 37 °C and 5% CO_2 for 4 days and examined for virus-induced cytopathic effects (CPE) using an inverted microscope. The $TCID_{50}$ was calculated using the Spearman and Kärber algorithm (Hierholzer & Killington, 1996).

Serum Neutralization Assays

Serial 1:2 dilutions of serum samples prepared in maintenance media were placed in replicate wells of a 96-well plate to which BVDV-1a Singer strain virus was added to a final virus concentration of 100-300 ($TCID_{50}$)/ml. Following a one-hour incubation period at 37 °C and 5% CO_2, well contents were transferred to 96-well tissue culture plates of approximately 80% confluent BT cells. Plates were incubated for 4 days at 37 °C and 5% CO_2 and examined

daily for the presence of virus-induced CPE using an inverted microscope. The endpoint neutralization titer was determined as the highest dilution without virus-induced CPE. Replicate wells containing neither serum nor virus were used as negative controls to monitor for virus contamination. Serum from an alphavirus ASFV HAp72-immunized pig was serially diluted 1:2 alongside the CSFV and BVDV serum samples as a control for non-specific antibody neutralization. To confirm the concentration of virus, the virus at the working dilution was back titrated using four 10-fold serial dilutions of the virus in maintenance media. The virus at the working dilution and the four serial dilutions were added to the 96-well serum neutralization plates in replicate wells.

Expression of CSFV and BVDV Polypeptides

Polypeptide fragments of BVDV and CSFV E2 were expressed using bacterial stocks of BVDV-1a Singer strain and CSFV C-strain E2 fragments that had been cloned into the histidine-tagged ubiquitin expression vector, pHUE, and transformed into the BL21 *E. coli* strain (New England Biolabs) using methods previously described (Trible *et al.*, 2011). The whole CSFV E2 and BVDV E2 proteins were divided into four non-overlapping polypeptide fragments designated as CSFV E2 1/4, 84 amino acids in length; 2/4, 84 amino acids; 3/4, 84 amino acids; and 4/4, 121 amino acids; or BVDV E2 1/4, 90 amino acids; 2/4, 90 amino acids; 3/4, 90 amino acids; and 4/4, 104 amino acids (Figure 2.1).

Overnight cultures were created by adding 7 µl of ampicillin (AMRESCO) to 7 ml of lysogeny broth (LB) medium (MP Biomedicals) and then adding a small inoculum of the BL21 *E. coli* strain containing the plasmid of interest. The overnight cultures were incubated for 12-18 hours at 37 °C in a bacterial shaking incubator. Culture volumes were increased by inoculating flasks containing 100 ml of prewarmed LB medium with 2-5 ml of the overnight

cultures and continuing to incubate them until the optical density of the samples measured at a wavelength of 600 nm (OD600) reached a value between 0.4-0.7. Cultures were then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (LabScientific) at a concentration of 0.1 M and then returned to the shaking incubator at 37 °C. The cultures were incubated for 4 hours post induction with IPTG and then centrifuged for 10 minutes at 4,000 x g. The supernatant was discarded and the resulting bacterial pellets were stored at -20 °C.

Native Purification of CSFV and BVDV Polypeptides

Bacterial pellets were resuspended in 5 ml of lysis equilibrium wash (LEW) buffer (Affymetrix). Following this, 50 µl of protease inhibitor cocktail (PIC) (Thermo Scientific) and 5 mg lysozyme (Fisher Scientific) were added to the resuspended lysate. The lysate was shaken on ice for 30 minutes and then sonicated using an ultrasonic homogenizer (BioLogics) at 40% power and 50% pulse for 2 minutes to lyse the cells. The lysate was centrifuged at 13,000 rpm for 30 minutes to recover the soluble protein fraction. The resulting supernatant was collected and filtered through a 0.45 µl filter to eliminate any insoluble material and the remaining pellet was stored at -20 °C. The supernatant was purified by nickel affinity chromatography using a Ni-IDA column (Affymetrix), as specified by the manufacturer, to bind the histidine tag from the pHUE vector. The flow through, washes, and elution fractions were collected and stored at 4 °C and an aliquot of each was analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were measured using a bovine serum albumin (BSA) assay.

Insoluble proteins were purified using the mild detergent 3-[cyclohexylamino]-1-propane-sulfonic acid (AMRESCO) and sodium lauroyl sarcosinate (Sigma-Aldrich) (CAPS/sarkosyl). The purified pellet from the soluble protein purification was resuspended in

10 ml of 0.5 M CAPS buffer with 0.3% sarkosyl and placed in a 37 °C water bath for 15 minutes. The resuspended pellets were frozen at -80 °C. Once frozen, the suspension was thawed at 37 °C for 15 minutes and refrozen at -80 °C. The freeze/thaw process was repeated two additional times and the final thawed suspension was centrifuged at 10,000 x g for 30 minutes. The resulting supernatant was filtered using a 0.25 µl filter to eliminate any insoluble material. Proteins were purified using nickel affinity chromatography as previously described for the soluble protein purification using 0.5 M CAPS buffer and 0.3% sarkosyl buffer instead of LEW and 0.5 M CAPS buffer with 0.3% sarkosyl and 250 mM imidazole (Fisher Scientific) buffer instead of elution buffer. The flow through, washes, and elution fractions were collected and stored at 4 °C and an aliquot of each was analyzed via SDS-PAGE. Protein concentrations were measured a using a BSA assay.

Western Blots

Western blot analysis was performed using the purified polypeptide fragments, CSFV E2 fragments 2/4 and 3/4, and all four BVDV E2 fragments. The proteins were transferred from an SDS-PAGE gel to a polyvinylidene difluoride (PVDF) (GE Healthcare) membrane by tank electroblotting and subsequently blocked overnight at 4 °C in 5% non-fat dry milk in PBS with added Tween 20 (PBS-T) (Fisher Scientific). The membrane was washed three times with PBS-T. Polyclonal serum from an alphavirus CSFV E2-immunized pig (pig 30) was diluted 1:1000 in 5% non-fat milk blocking solution and added to the membrane. Following a one-hour incubation period, the membrane was washed three times with PBS-T. Goat anti-porcine IgG antibody conjugated to horseradish peroxidase (HRP) (ICN Biomedicals) was diluted 1:2000 in a 5% non-fat milk blocking solution and added to the membrane. Following a one-hour incubation period, the membrane was washed three times with PBS-T. Visualization was performed using a

commercial peroxidase kit (Pierce DAB substrate kit, Thermo Fisher Scientific). The DAB substrate solution was added to the membrane and incubated for 20 minutes. Development was stopped by the addition of ddH_2O to the membrane.

Tables and Figures

Figure 2.1 CSFV and BVDV pHUE Fragment Constructs

Polypeptide fragments of CSFV E2 and BVDV E2 that were cloned into the pHUE expression vector and expressed in BL21 *E. coli* strain bacteria. For both viruses, the whole E2 protein was broken down into four different polypeptide fragments of about equal size with no overlapping amino acids. The fragment length in amino acids is provided in the column on the left and the corresponding beginning and ending amino acids of the whole polyprotein to which the fragment corresponds are indicated on the bottom of each fragment. CSFV amino acid positons are in reference to CSFV C-strain GenBank accession number Z46258.1-1 and BVDV amino acids positions are in reference to BVDV-1a Singer strain GenBank accession number DQ0889995.

CSFV E2 Fragments

Fragment	Length (aa)	
Whole	373	690 — 1062
1/4	84	690 — 773
2/4	84	774 — 857
3/4	84	858 — 941
4/4	121	942 — 1062

BVDV E2 Fragments

Fragment	Length (aa)	
Whole	374	693 — 1066
1/4	90	693 — 782
2/4	90	783 — 872
3/4	90	873 — 962
4/4	104	963 — 1066

Chapter 3 - Results

Comparison of CSFV and BVDV E2 and E^{rns}

The antigenic targets this work focuses on consist of CSFV and BVDV glycoproteins E2 and E^{rns}. The identity shared between BVDV and CSFV, specifically for these two glycoproteins, is responsible for the production of antibodies with cross-reactive and cross-neutralizing activity. A pairwise comparison of C-strain CSFV E2 and Singer strain BVDV-1a E2, the glycoprotein responsible for viral binding and entry into the host cell and against which antibodies are made, reveals a shared identity of 59.36% between the glycoproteins (Figure 3.1). Pairwise comparison of CSFV E^{rns} and BVDV E^{rns}, the other glycoprotein against which antibodies are made, reveals a shared identity of 73.13% (Figure 3.2). These comparisons show moderate identity between the CSFV and BVDV glycoproteins E2 and E^{rns}; a number of shared runs of amino acids are shown when their sequences are aligned. To determine the location on CSFV and BVDV to which cross-reactive and cross-neutralizing antibodies react, serum neutralization assays were performed to confirm the presence of cross-neutralizing antibodies produced in response to alphavirus vaccine immunization.

Serum Neutralization Assays

The purpose of this work was to determine if antibodies produced by swine in response to immunization with an alphavirus vaccine construct possess neutralizing activity against a cytopathic BVDV strain. Prior to performing any serum neutralization assays, BVDV-1a Singer strain stock virus was propagated in BT cells and collected on day 6 when more than 70% of the cells showed varying signs of cytopathic effects (CPE). The $TCID_{50}$ of this stock virus was calculated to be 2.22×10^6 $TCID_{50}$/ml. Serum neutralization assays were performed in replicate wells of 96-well tissue culture plates using serial dilutions of polyclonal swine serum from five

animals immunized with BVDV-1b E2, four animals immunized with CSFV E2, and four animals immunized with CSFV E^{rns}. The BVDV E2 samples served as a positive control to which cross-neutralization activity of the CSFV E2 and E^{rns} samples could be compared. Replicate wells with no added virus served as negative controls to monitor for virus contamination. Serial dilutions of serum from an ASFV p72-immunized animal were used as negative controls to monitor for non-specific antibody neutralization. The diluted serum samples were incubated with 100-300 $TCID_{50}$ of BVDV-1a Singer strain virus and transferred to plates of approximately 80% confluent BT cells. Back titrations using the virus at working dilution were also performed on the serum neutralization plates as a confirmation of the $TCID_{50}$/ml. The plates were observed daily for CPE. CPE characteristic of BVDV infection (Figure 3.3) were first observed in 90% of infected wells 3 days after viral inoculation with significant CPE throughout the well by day 4. Neutralization titers were calculated as the highest dilution of serum in which CPE was not observed (Table 3.1). The negative control wells were observed and remained free of CPE and the wells containing the ASFV p72-immunized pig serum developed signs of CPE as expected. The results of these assays demonstrated neutralizing titers for the serum samples from animals immunized with the BVDV-1b E2 and CSFV E2 and E^{rns} alphavirus constructs.

The first serum neutralization assay performed showed that the animals immunized with the BVDV and CSFV alphavirus constructs had detectable neutralizing titers. Of the three alphavirus constructs used for immunization, those animals vaccinated with the BVDV-1b construct had the highest neutralizing titers. Serum from animals 11, 12, 13, and 15 was able to neutralize the virus at all dilutions (1:8 through 1:1024) and only one sample from an animal immunized with BVDV-1b, pig 14, was not able to completely neutralize the virus at all dilutions with a neutralization titer of 1:512. Serum from three animals immunized with the

CSFV E2 construct, pigs 21, 23, and 29, was used for the assay and had low neutralization titers. Samples from animals 21 and 23 demonstrated no neutralizing activity at any dilution while serum from pig 29 had a neutralization titer of 1:16. The two CSFV E^{rns} samples used for the assay, serum from pig 41 and 47, both showed neutralizing activity. Serum from pig 41 had a neutralizing titer of 1:8 and serum from pig 47 had neutralizing titers ranging from 1:16 to 1:32. The results of this assay showed that antibodies generated in response to immunization with an alphavirus BVDV-1b construct can neutralize BVDV-1a virus and that both CSFV E2 and E^{rns} serum samples possess cross-neutralizing antibodies.

A second serum neutralization assay was performed to better characterize the neutralizing activity of the samples. Using the same set of serum samples, serum dilutions were performed starting at a 1:32 dilution. Neutralization titers of serum from animals immunized with the BVDV-1b construct differed from the first assay in that serum from animals 11, 12, and 14 had lower titers than were observed previously. The titers observed for serum from animals 13 and 15 confirmed the results of the first assay, as they were 1:1024 and 1:2048, respectively. The CSFV E2 samples had similar titers as observed in the previous assay with serum from animals 21 and 23 showing no neutralizing activity. Serum from animal 29 had one replicate with neutralizing activity at the lowest dilution (1:32) and the other with no neutralizing activity (less than 1:32). The serum from animals immunized with the CSFV E^{rns} construct had similar results to the first assay with neutralization occurring at the lowest dilution for serum from pig 41 and no neutralizing activity observed for serum from pig 47. The titers collected from this assay confirmed the previously observed neutralizing activity of BVDV-1b samples from animals 13 and 14 and low neutralizing activity of samples 29 and 41.

A third assay was performed to check the concentration of the virus at working dilution using serum samples from two animals that previously demonstrated neutralizing activity, pig 12 immunized with the BVDV-1b construct and pig 47 immunized with the CSF E^{rns} construct. Results similar to the first neutralization assay were observed for both animals with serum from pig 12 demonstrating complete neutralizing activity for all dilutions (1:8 through 1:1024) and neutralizing titers ranging from 1:16 to 1:32 for serum from pig 47. This assay confirmed the high titers seen in the first assay for serum from pig 12 and low, yet detectable, neutralization activity for serum from pig 47. The concentration of the virus at the working dilution was also confirmed to be correct.

The fourth serum neutralization assay was performed utilizing all of the serum samples used previously in assays one and two. Four of the five serum samples from BVDV-1b-immunized animals were completely neutralizing at all dilutions (1:8 through 1:1024). Only one sample, serum from pig 13, did not show complete neutralization and had neutralizing titers ranging from 1:256 through greater than 1:1024. Of the three CSFV E2 samples, only serum from pig 29 showed neutralizing activity with titers ranging from no neutralizing activity (less than 1:8) through 1:32. The two CSFV E^{rns} samples showed some neutralizing activity with titers for serum from pig 41 ranging from 1:8 through 1:32 and ranging from 1:16 through 1:32 for serum from pig 47. The results of this assay were similar to those seen for assays one and two, with the exception of serum from pig 13, which had lower titers than were observed previously.

Finally, a fifth serum neutralizations assay was performed using only serum from animals immunized with the CSFV E2 and E^{rns} constructs. Two CSFV E2 samples, obtained from pigs 21 and 30, and four CSFV E^{rns} samples, obtained from pigs 41, 46, 47, and 48, were used for the assay. Both CSFV E2 samples had neutralizing activity ranging from 1:16 to 1:32 and two of the

CSFV E^{rns} serum samples, from pigs 46 and 48, had no neutralizing activity (less than 1:8). Serum from pig 41 had low neutralizing titers ranging from no neutralizing activity to 1:8 and pig 47 serum had similar neutralizing titers ranging from no neutralizing activity to 1:16. The results of this assay showed no neutralizing activity for two previously untested CSFV E^{rns} samples, serum from pigs 46 and 48; similar neutralizing activity for serum from pigs 41 and 47; and some neutralizing activity for CSFV E2 with serum from pig 21.

The results of this work, five assays over the course of 10 months, showed high neutralizing titers for serum from swine immunized with the alphavirus BVDV-1b E2 construct against the homologous virus and weak, but detectable, neutralizing titers for the CSFV E2 and E^{rns} samples of the heterologous virus, demonstrating the presence of cross-neutralizing antibodies. Of the five BVDV-1b samples that were used, serum from pig 15 had high titers that were reproducible and had the highest neutralizing titer out of all of the BVDV samples with a titer of 1:2048. The other three BVDV-1b samples that were examined had titers that ranged from 1:64 to greater than 1:1024 with a majority of the replicates showing strong neutralizing activity with titers of 1:1024 or greater than 1:1024. The neutralizing titers of the four CSFV E2 and four CSFV E^{rns} samples were significantly lower than those observed for BVDV-1b samples. Neutralization titers for these animals ranged from 1:8 to 1:32 with no neutralizing activity observed for animals 23, 46, or 48 and some detectable activity for animals 21, 29, 30, 41, and 47. The variation observed in titers among the assays may be due to the varying amount of virus used in each assay. Overall, this work identified the presence of BVDV-1b E2, CSFV E2, and CSFV E^{rns} antibodies generated in response to alphavirus vector immunization with neutralizing activity for BVDV-1a.

Expression and Purification of CSFV and BVDV E2 Polypeptide Fragments

Based on the results of the serum neutralization assays, the next aim of this study was to map the antigenic regions to which the antibodies reacted. The purpose of this work was to express and purify polypeptide fragments of CSFV E2 and BVDV E2 for use in western blot analysis to map the antigenic regions where the antibodies against CSFV E2 and BVDV E2 bind. Four polypeptide fragments of BVDV E2 and two polypeptide fragments of CSFV E2 were successfully expressed using the pHUE expression vector in BL21 strain *E. coli* cells and purified under native conditions using affinity chromatography.

All four polypeptide fragments of BVDV E2 were successfully expressed and purified under native conditions using CAPS/sarkosyl. Protein concentrations were 322.3 µg/ml, 359.2 µg/ml, 204.4 µg/ml, and 126.2 µg/ml for fragments 1/4, 2/4, 3/4 and 4/4, respectively (Table 3.2). Protein purity was analyzed using SDS-PAGE. A single band was visualized for each of the four polypeptide fragments with no additional bands detected (Figure 3.4). BVDV E2 fragment 4/4 had the faintest band due to the lower concentration of the expressed product.

Three of the CSFV E2 polypeptide fragments, 1/4, 2/4 and 3/4, were successfully expressed and purified under native conditions. CSFV E2 fragments 1/4 and 3/4 were purified using CAPS/sarkosyl and fragment 1/4 was purified using a standard native protocol. Protein purity was analyzed using SDS-PAGE and a single band was visualized for each fragment (Figure 3.4). Protein concentrations were 210 µg/ml and 224 µg/ml for CSFV E2 fragments 2/4 and 3/4, respectively (Table 3.2).

Although CSFV E2 fragment 1/4 was successfully expressed and purified, the protein concentration was too low for use in the remainder of the study. The whole CSFV E2 protein and CSFV E2 fragment 4/4 were not available for further use in the study as these were never

successfully expressed, likely due to the presence of the C-terminal transmembrane region. The whole BVDV E2 protein was not successfully expressed and was also not available for further use in the study. The successful expression and purification of all four BVDV E2 fragments and CSFV E2 fragments 2/4 and 4/4 was sufficient to map the antigenic regions of the CSFV E2 and BVDV E2 antibodies.

Western Blot

Following the expression and purification of the six CSFV E2 and BVDV E2 polypeptide fragments, western blot analysis was performed for the purpose of mapping the antigenic binding regions of the CSFV E2 and BVDV E2 antibodies in the polyclonal serum from the alphavirus-immunized swine. The western blot was performed using CSFV E2 fragments 2/4 and 3/4 and all four BVDV E2 fragments with polyclonal serum from alphavirus CSFV E2-immunized pig 30. The CSFV E2 fragments were used as positive controls to identify the region the antibodies were reacting against on the homologous protein. The BVDV E2 fragments were used to identify the region of the heterologous protein for which cross-reactivity of the antibodies had been demonstrated. The results of this work showed that antibodies generated in response to immunization with an alphavirus CSFV E2 vaccine construct possess cross-reactive activity for two regions on the BVDV E2 protein.

All proteins that were visualized on the resulting western blot had single bands of expected size (Figure 3.5). The serum from the alphavirus CSFV E2-immunized pig reacted with both CSFV E2 fragments 2/4 and 3/4. CSFV E2 fragment 2/4 had the most prominent band while CSFV fragment 3/4 had a faint band. BVDV E2 fragments 1/4 and 2/4 were also recognized by the serum resulting from vaccination with the alphavirus CSFV E2 construct and had faint bands comparable to that seen for CSFV E2 3/4. This same serum did not recognize

BVDV E2 fragments 3/4 and 4/4 as no bands were observed. However, both CSFV E2 fragment 2/4 and BVDV E2 fragment 2/4 were recognized by this serum suggesting that the cross-reactive antibodies between the two pestiviruses recognize one or multiple epitopes that are on these fragments. The results of this work demonstrate that the polyclonal serum from the animal immunized with the alphavirus CSFV E2 construct, pig 30, recognized both CSFV E2 and BVDV E2 protein fragments and indicates the presence of cross-reactive antibodies between BVDV E2 and CSFV E2 that recognize CSFV amino acid positions 774 through 857 and BVDV amino acid positions 783 through 872.

Tables and Figures

Table 3.1 Summary of Results of Serum Neutralization Assays

Assays and Neutralization Titers															
Pig Number	Vaccine Construct	Assay 1		Assay 2		Assay 3				Assay 4			Assay 5		
		1	2	1	2	1	2	3	4	1	2	3	1	2	3
11	BVDV E2	> 1:1024	> 1:1024	1:128	1:256	-	-	-	-	> 1:1024	> 1:1024	> 1:1024	-	-	-
12	BVDV E2	> 1:1024	> 1:1024	1:512	1:1024	> 1:1024	> 1:1024	> 1:1024	> 1:1024	> 1:1024	> 1:1024	> 1:1024	-	-	-
13	BVDV E2	> 1:1024	> 1:1024	1:1024	1:1024	-	-	-	-	> 1:1024	1:512	1:256	-	-	-
14	BVDV E2	1:512	1:512	1:64	1:64	-	-	-	-	> 1:1024	> 1:1024	> 1:1024	-	-	-
15	BVDV E2	> 1:1024	> 1:1024	1:2048	1:2048	-	-	-	-	> 1:1024	> 1:1024	> 1:1024	-	-	-
21	CSFV E2	< 1:8	< 1:8	< 1:32	< 1:32	-	-	-	-	< 1:8	< 1:8	< 1:8	1:32	1:16	1:32
23	CSFV E2	< 1:8	< 1:8	< 1:32	< 1:32	-	-	-	-	< 1:8	< 1:8	< 1:8	-	-	-
29	CSFV E2	1:16	1:16	1:32	< 1:32	-	-	-	-	< 1:8	1:32	1:32	-	-	-
30	CSFV E2	-	-	-	-	-	-	-	-	-	-	-	1:16	1:16	1:32
41	CSFV E^{rns}	1:8	1:8	1:32	< 1:32	-	-	-	-	1:16	1:32	1:8	1:8	< 1:8	< 1:8
46	CSFV E^{rns}	-	-	-	-	-	-	-	-	-	-	-	< 1:8	< 1:8	< 1:8
47	CSFV E^{rns}	1:32	1:16	< 1:32	< 1:32	1:32	1:16	1:32	1:8	1:16	1:16	1:32	< 1:8	1:16	1:16
48	CSFV E^{rns}	-	-	-	-	-	-	-	-	-	-	-	< 1:8	< 1:8	< 1:8

Note: Serum neutralization assays were performed with serum samples in replicate wells on each plate and the neutralization titer for each replicate is provided. Assays one and two were performed in duplicate, assay three was performed in quadruplicate, and assays four and five were performed in triplicate. Neutralization titers with a greater than symbol (>) indicate that complete neutralization was observed through the highest dilution for that assay for that replicate. Titers with a less than symbol (<) indicate no neutralization was observed at the lowest dilution for that assay for that replicate. A minus symbol (-) indicates that no data was collected for that serum sample.

Table 3.2 CSFV and BVDV Polypeptide Fragment Purification Summary

Fragment	Bacterial Stock Number	Purification Method	Size (kDa)	Protein Concentration (µg/mL)
CSFV E2 Whole	3127	-	50	-
CSFV E2 1/4	3131	CAPS/sarkosyl	18	-
CSFV E2 2/4	3132	CAPS/sarkosyl	18	210
CSFV E2 3/4	3133	Native	18	224
CSFV E2 4/4	3126	-	22	-
BVDV E2 Whole	3216	-	50	-
BVDV E2 1/4	3214	CAPS/sarkosyl	18	322.3
BVDV E2 2/4	3221	CAPS/sarkosyl	18	359.2
BVDV E2 3/4	3222	CAPS/sarkosyl	18	204.4
BVDV E2 4/4	3223	CAPS/sarkosyl	19.5	126.2

Note: No purification method or protein concentration is given for CSFV E2 whole, CSFV E2 fragment 4/4, or BVDV E2 whole as these polypeptides were never successfully expressed or purified using the described vector system.

Figure 3.1 Protein Sequence Alignment of CSFV E2 and BVDV E2

Protein sequence alignment of the E2 glycoproteins of CSFV C-strain (GenBank accession number Z46258.1-1) and BVDV-1a Singer strain (GenBank accession number DQ088995.2) performed using CLC Main Workbench. The numbers at the end of each alignment indicate the amino acid positions within the polyprotein. Hyphens (-) located in the sequence rows indicate gaps in the alignment. Asterisks (*) located in the consensus row indicate non-consensus positions.

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                                    20                                  40
CSFV C-strain E2          RLACKEDYRY AISSTDEIGL LGAGGLTTTW KEYNHDLQLN DGTVKASCVA 50
BVDV-1a Singer strain E2  DLDCKPEFSY AIARDERIGQ LGAEGLTTTL EGLLAEMKLE DTMVIAWCKD 50
Consensus                 *L*CK****Y AI*****IG* LGA*GLTTT* ********L* D**V*A*C**
                          60                                  80                  100
CSFV C-strain E2          GSFKVTALNV VSRRYLASLH KKALPTSVTF ELLFDGTNPS -TEEMGDDFR 99
BVDV-1a Singer strain E2  GKFTYLPRCT RETRYLAILH TRALPTSVVF KKLFDGRKQE DVVEMDDNFE 100
Consensus                 G*F******* ***RYLA*LH **ALPTSV*F **LFDG**** D**EM*D*F*
                                   120                                 140
CSFV C-strain E2          SGLCPFDTSP VVKGKYNTTL LNGSAFYLVC PIGWTGVIEC TAVSPTTLRT 149
BVDV-1a Singer strain E2  FGLCPCDAKP IVRGKFNTTL LNGPAFQMVC PIGWTGTVSC MSFNMDTLAT 150
Consensus                 *GLCP*D**P *V*GK*NTTL LNG*AF**VC PIGWTG***C ******TL*T
                          160                 180                 200
CSFV C-strain E2          EVVKTFRRDK PFPHRMDCVT TTVENEDLFY CKLGGNWTCV KGEPVVYTGG 199
BVDV-1a Singer strain E2  TVIRTYRRSK PFPHRQGCIT QKTLGEDLHN CILGGNWTCV PGDMLLYKGG 200
Consensus                 *V**T*RR*K PFPHR**C*T *****EDL** C*LGGNWTCV *G****Y*GG
                                   220                                 240
CSFV C-strain E2          LVKQCRWCGF DFDGPDGLPH YPIGKCILAN ETGYRIVDST DCNRDGVVIS 249
BVDV-1a Singer strain E2  SIESCKWCGY QFKESEGLPH YPIGKCRLEN ETGYRLVDDT SCNREGVAIV 250
Consensus                 ****C*WCG* *F****GLPH YPIGKC*L*N ETGYR*VD*T *CNR*GV*I*
                          260                 280                 300
CSFV C-strain E2          TEGSHECLIG NTTVKVHASD ERLGPMPCRP KEIVSSAGPV KKTSCTFNYT 299
BVDV-1a Singer strain E2  PQGILRCKIG KTTIQVIAMD TKLGPMPCRP YEIISSEGPV ERTACTFNYT 300
Consensus                 **G***C*IG *TT**V*A*D **LGPMPCRP *EI*SS*GPV **T*CTFNYT
                                   320                                 340
CSFV C-strain E2          KTLKNRYYEP RDSYFQQYML KGEYQYWFDL DATDRHSDYF AEFVVLVVVA 349
BVDV-1a Singer strain E2  KTLKNKYFEP RDSYFQQYML KGEYQYWFDL EVTDHHRDYF AESILVVVVA 350
Consensus                 KTLKN*Y*EP RDSYFQQYML KGEYQYWFDL **TD*H*DYF AE****VVVA
                          360
CSFV C-strain E2          LLGGRYVLWL IVTYVVLTEQ LAAG 373
BVDV-1a Singer strain E2  LLGGRYVLCL LVTYMVLSEQ KASG 374
Consensus                 LLGGRYVL*L *VTY*VL*EQ *A*G
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Figure 3.2 Protein Sequence Alignment of CSFV E^{rns} and BVDV E^{rns}

Protein sequence alignment of the E^{rns} glycoproteins of CSFV C-strain (GenBank accession number Z46258.1-1) and BVDV-1a Singer strain (GenBank accession number DQ088995.2) performed using CLC Main Workbench. The numbers at the end of each alignment indicate the amino acid positions within the polyprotein. Hyphens (-) located in the sequence rows indicate gaps in the alignment. Asterisks (*) located in the consensus row indicate non-consensus positions.

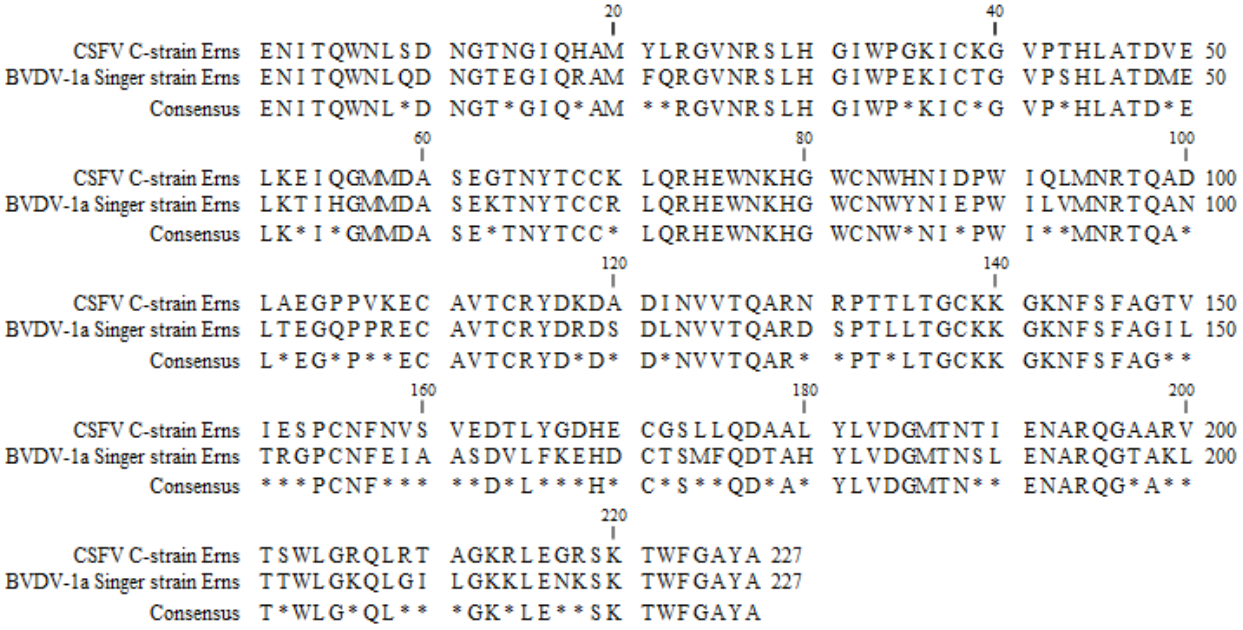


Figure 3.3 BT Cells in Cell Culture

Panel A shows healthy BT cells at approximately 80% confluency. Panel B shows BT cells infected with BVDV-1a Singer strain virus displaying CPE characteristic of BVDV infection. Increasing vacuolization, clumping of apoptotic cells, and detachment from the flask are characteristic of BVDV infected BT cells.

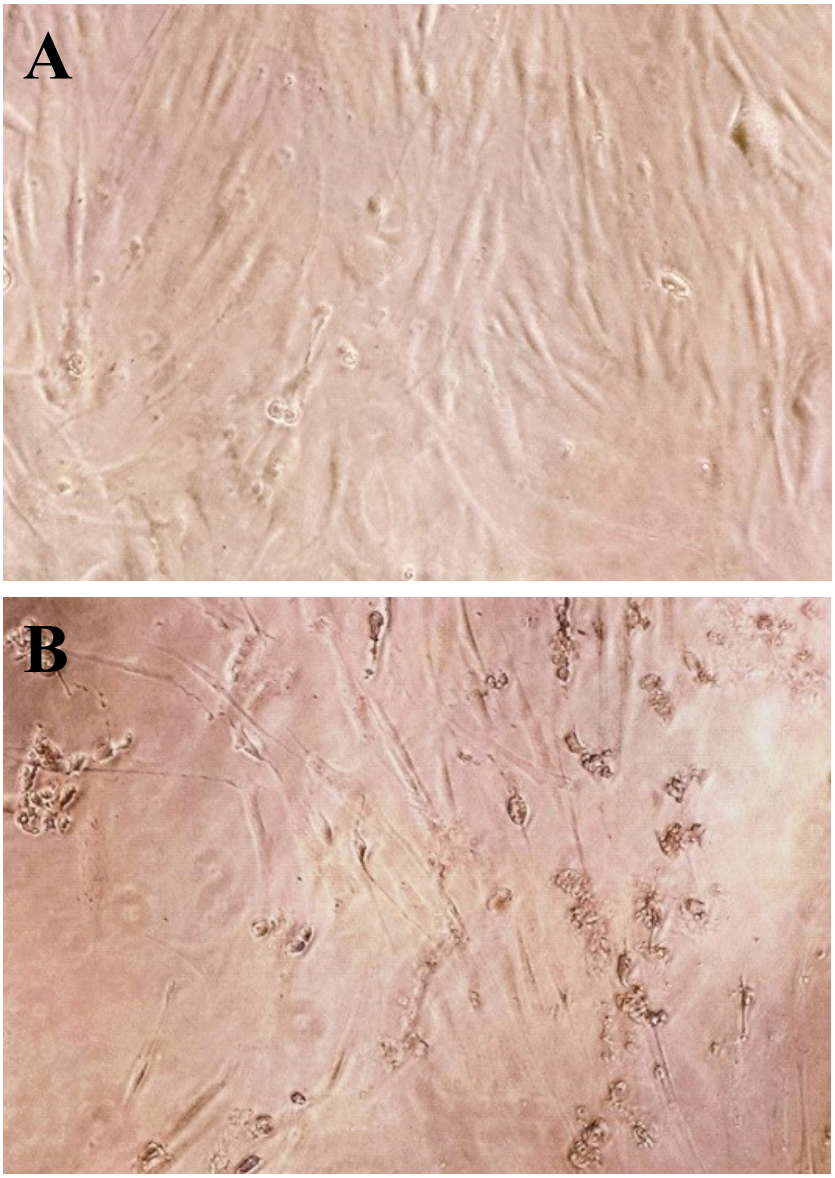


Figure 3.4 SDS-PAGE Gel of CSFV and BVDV E2 Polypeptide Fragments Used For Western Blot

Standard SDS-PAGE gel stained with SimplyBlue™ SafeStain showing the purification products of CSFV E2 and BVDV E2 polypeptide fragments used to generate the western blot in Figure 3.3. Lane one contains the Precision Plus Protein Kaleidoscope Prestained Protein Standards with the 15 and 20 kDa bands identified. Lane two contains CSFV E2 fragment 2/4, lane three contains CSFV E2 fragment 3/4, lane four contains BVDV E2 fragment 1/4, lane five contains BVDV E2 fragment 3/4, lane six contains BVDV E2 fragment 2/4, and lane seven contains BVDV E2 fragment 4/4. Note that BVDV E2 fragment 2/4 and BVDV E2 fragment 3/4 are switched on the SDS-PAGE gel as compared to the western blot.

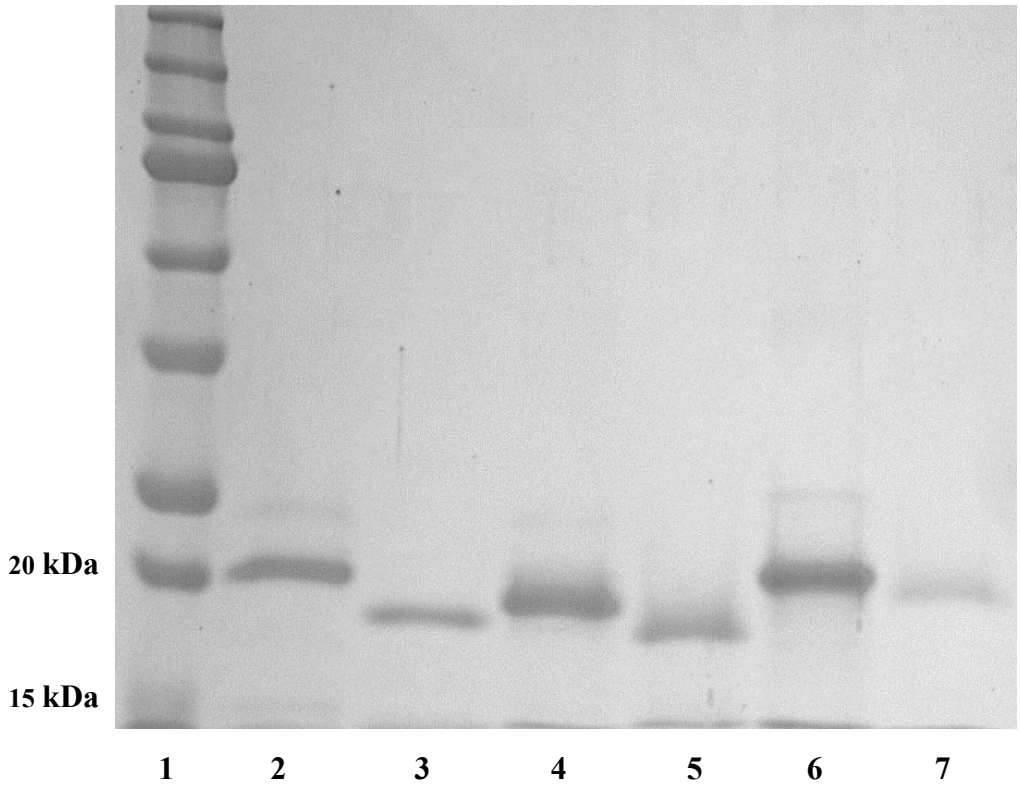
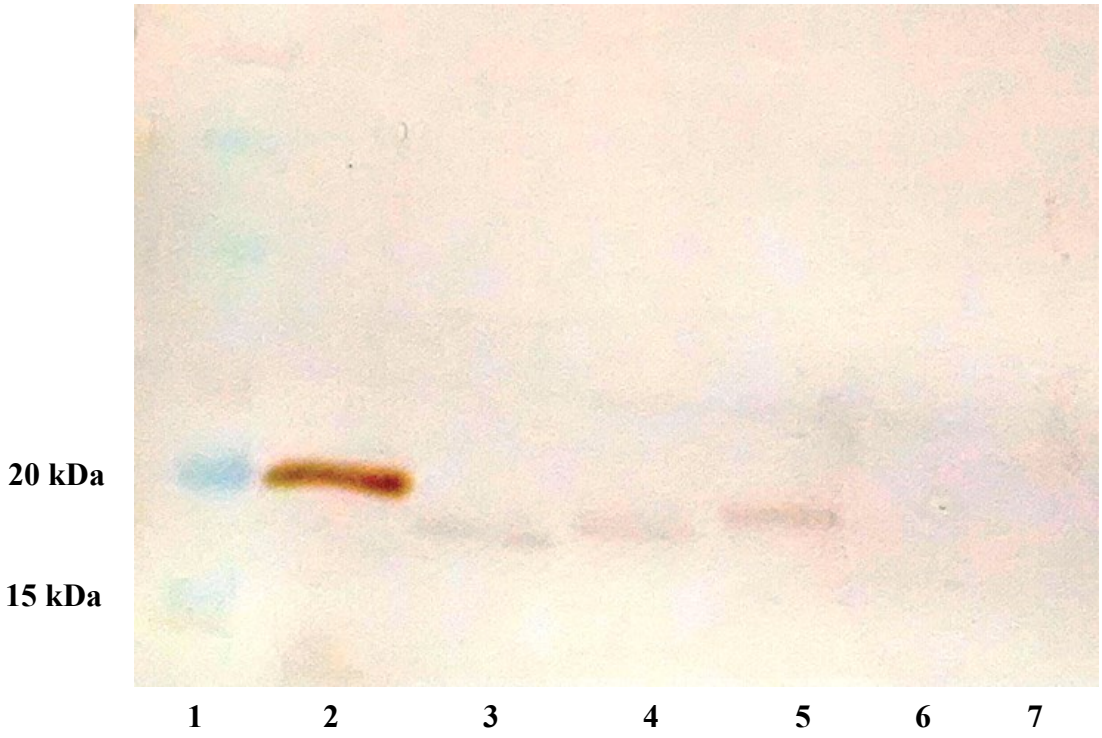


Figure 3.5 Western Blot of CSFV and BVDV E2 Polypeptide Fragments

Western blot performed using purified CSFV E2 fragments 2/4 and 4/4 and BVDV E2 fragments 1/4, 2/4, 3/4, and 4/4 reacted against polyclonal swine serum from pig 30 obtained following immunization with the CSFV E2 alphavirus construct and goat anti-porcine HRP conjugated secondary antibody. Visualization was achieved using the Thermo Scientific Pierce DAB substrate kit. Lane one shows the Precision Plus Protein Kaleidoscope Prestained Protein Standards with the 15 and 20 kDa bands identified. Lane two shows the strong reactivity of CSFV E2 fragment 2/4, lane three shows weak reactivity of CSFV E2 fragment 3/4, lane four shows weak reactivity of BVDV E2 fragment 1/4, lane five shows weak reactivity of BVDV E2 fragment 2/4, and lanes six and seven show no reactivity of BVDV E2 fragments 3/4 and 4/4 to the serum from the pig immunized with the alphavirus CSFV E2 construct.



Chapter 4 - Discussion and Conclusions

The objective of this study was to determine the epitope regions of cross-reactivity between the CSFV E2 and BVDV E2 glycoproteins as a means to identify epitopes that differ between the two pestivirus species. Serum neutralization assays performed using serum from groups of pigs immunized with an Alphavirus expressing either BVDV-1b E2, CSFV E2, or CSFV E^{rns} demonstrated neutralizing activity towards BVDV-1a *in vitro*. Additionally, a western blot using serum from a pig immunized with the Alphavirus CSFV E2 viral vector construct recognized both CSFV E2 and BVDV E2 polypeptide fragments. The results of this study identified CSFV amino acid positions 774 through 857 and BVDV amino acid positions 783 through 872 as the regions that contain the epitopes recognized by cross-reactive antibodies between BVDV E2 and CSFV E2.

Early research of pestiviruses utilized similar neutralization assays using antiserum and monoclonal antibodies to first identify BVDV infection in swine and to better characterize pestivirus strains. One of first observances of BVDV in swine was identified in laboratory animals that had antibody titers of 1:4 and 1:16 against CSFV and 1:256 and 1:1024 against BVDV (Carbrey *et al.*, 1969, 1976). To better characterize the pestivirus groups, studies using anti-serum against multiple pestivirus strains collected from cattle, swine, and sheep were tested for their neutralizing activity against a number of pestivirus isolates. Neutralizing titers were greatest against homologous species with observed cross-neutralization of almost all heterologous species, generally at much lower titers (Dekker *et al.*, 1995; Wensvoort *et al.*, 1989b). To account for this observed cross-neutralization between pestivirus species, panels of monoclonal antibodies were developed for differentiation of CSFV. Monoclonal antibodies that

were pan-pestivirus specific, detecting most strains of CSFV, BVDV, and BDV were identified in addition to those monoclonal antibodies specific for only CSFV, suggesting that the CSFV-specific monoclonal antibodies detected a conserved region of CSFV that is not found in the other pestiviruses (Edwards *et al.*, 1991; Wensvoort *et al.*, 1989a).

Serum neutralization titers calculated in this study showed that the serum taken from pigs immunized with a BVDV E2 viral construct had the highest neutralization titers against BVDV and lower titers for the CSFV E2 and E^{rns} constructs that ranged from not detectable (less than 1:8) to 1:32. The resulting lower neutralization titers for CSFV E2 and E^{rns} were expected as only cross-protective antibodies are capable of neutralizing BVDV in this assay, accounting for a fraction of the total antibody population. Neutralization assays to measure the activity of the antibodies to CSFV could not be performed in a BSL-2 laboratory, as live CSFV is a BSL-3 agent, and was therefore not an option for this study. The high neutralization titers to homologous virus strains and the low neutralization titers to heterologous virus strains reflect the findings of earlier pestivirus research (Carbrey *et al.*, 1976; Dekker *et al.*, 1995; Wensvoort *et al.*, 1989b).

Eight polypeptide fragments of CSFV E2 and BVDV E2 were expressed using the pHUE plasmid expression vector system in *E. coli*. Fragments 2/4 and 3/4 of CSFV E2 and all four fragments of BVDV E2 were successfully expressed and purified. Although CSFV E2 fragment 1/4 was successfully expressed, the purification resulted in protein concentrations that were too low to be used for the rest of the study. The western blot results utilizing these purified fragments showed strong reactivity to CSFV E2 2/4 (amino acids 774-857) which encompasses all of domain A (amino acids 766-866), as well as the C-terminals of domains B and C (amino acids 691-773 and 691-800, respectively) (Figure 4.1). Epitopes that have been previously mapped to

this region include ^{829}TAVSPTTLR^{837}, which is conserved among CSFV strains (Lin *et al.*, 2000). CSFV E2 fragment 3/4 (amino acids 858-941), which represents only 9 amino acids of the C-terminal of domain A (amino acids 776-866), had weak reactivity (Figure 4.1). Although no known epitopes of CSFV have been mapped to this fragment, antibodies have been identified against domain A (Lin *et al.*, 2000; Nishimori *et al.*, 1996; van Rijn *et al.*, 1993).

BVDV E2 fragment 1/4 (amino acids 693-782), which encompasses domain I of BVDV E2 (amino acids 693-782), had weak reactivity similar to that of CSFV E2 3/4 (Figure 4.2). A previous study described an epitope found to have high homology between pestivirus species that lies on this fragment. Identified as a cluster of amino acids in the center of the 16 amino acid sequence ^{756}RYLAILHTRALPTSVV^{771} in BVDV-1 strain NADL, amino acids ^{763}T, ^{764}R, and ^{766}L on the C-terminus of BVDV E2 fragment 1/4 may be responsible for the weak reactivity seen on the western blot (Paton *et al.*, 1992a).

Interestingly, the motif ^{753}RYLASLHKKALPT^{765} was identified by another study as one that is shared among CSFV strains and also shares amino acids ^{753}R, ^{754}Y, ^{755}L, ^{756}A, ^{758}L, ^{759}H, ^{762}A, ^{763}L, ^{764}P, ^{765}T with BVDV-1 (Chang *et al.*, 2012b). Domain I of BVDV corresponds to antigenic unit B/C of CSFV. Previous studies have shown that antibodies made against the B/C domain of CSFV do possess neutralizing activity. It could therefore be hypothesized that the ^{753}RYLASLHKKALPT^{765} motif, as it lies on the B/C domain of CSFV, may be responsible for the cross-reactivity observed in western blot analysis and cross-neutralizing activity observed *in vitro* of antibodies produced in response to CSFV E2 of BVDV.

BVDV E2 fragment 2/4 (amino acids 783-872), which contains all of domain II (amino acids 783-860) and the N-terminal of domain III (amino acids 861-1035), also had weak reactivity on western blot similar to that of CSFV E2 3/4 and BVDV E2 1/4 (Figure 4.2). A

BVDV epitope, Z2, was mapped to amino acids ^{813}LNGPAFQMVCPIGTGTVSCTLANKDTLAT^{842} and represents an area of high homology between BVDV sequences (Jelsma *et al.*, 2013). This area, found at the center of the D/A domain of CSFV, is a region with high homology between BVDV that is also shared by CSFV. A potential epitope in which antibodies are generated that are cross-reacting and cross-neutralizing may lie in this region.

Further analysis of the Z2 epitope revealed that it corresponds to CSFV E2 domain A, amino acid positions 809-838, which is found on CSFV E2 fragment 2/4. In addition, a highly conserved CSFV epitope, ^{829}TAVSPTTLR^{837}, can also be mapped to this region (Lin *et al.*, 2000). However, this epitope is highly variable among strains of BVDV and BDV and has little homology with BVDV-1. When both E2 sequences are aligned, amino acids ^{809}NTTLLNGPAFQMVCPIGWTG^{828} of BVDV have 85% homology between CSFV C-strain and BVDV-1a Singer strain, which encompasses part of the Z2 epitope and lacks the unique CSFV ^{829}TAVSPTTLR^{837} epitope. While this identified region could represent a potential epitope to which antibodies are made that are cross-neutralizing and cross-reactive, there is currently no other data in the literature to suggest this.

Future work should be done to truncate the CSFV E2 protein to remove the transmembrane region, as the presence of this region on the C-terminus of CSFV E2 fragment 4/4 is likely the reason this protein was not successfully expressed in the pHUE *E. coli* vector. A sequence of 18 amino acids first identified at the C-terminus of strain Brescia, ^{1032}IVLVVVALLGGRYVLWLI^{1048}, which is likely the transmembrane domain that anchors E2 into the lipid bilayer of the viral envelope, may be responsible for the inability of this protein to be expressed (Moormann *et al.*, 1990). Previous studies that expressed CSFV E2 found that

expression levels were higher for E2 sequences that lacked this transmembrane region (Hulst *et al.*, 1993; van Rijn *et al.*, 1996). A hydrophobicity plot of CSFV C-strain E2 was generated to demonstrate the location of this hydrophobic transmembrane region (Figure 4.3).

Future efforts to develop serological assays with improved specificity for CSFV should be done to further refine the location of the epitopes responsible for cross-reactivity on CSFV E2 fragment 2/4 and examine CSFV E2 fragment 1/4 for cross-reactive epitopes. Once identified, these epitope locations should be avoided for use in the development of serological assays as they represent a potential area of cross-reactivity that would give a false-positive result for a CSFV-negative animal. The epitopes identified by these studies would also improve DIVA subunit vaccine constructs as prior infection and seroconversion to BVDV could inhibit or dampen the immune response to vaccination. Therefore, vaccines should contain epitopes that are not shared between the viruses and are unique to only CSFV to ensure that vaccination generates a protective immune response. A similar and more common example of this immunological event occurs in neonates and infants that possess maternal antibodies. Maternal antibodies present at the time of vaccination neutralize the antigen before the immune system can mount a response, thereby inhibiting the production of any form of immunological memory by the young animal. This form of passive immunity is the reason why immunizations are not administered before a certain age in most species (Niewiesk, 2014).

Mapping the cross-reactive epitopes of CSFV E^{rns} and BVDV E^{rns} should be performed for the development of improved serological assays. This is especially important as a number of CSFV DIVA vaccine candidates rely on vaccination with CSFV E2 and require a companion ELISA to detect for the presence of CSFV E^{rns}. One such marker vaccine candidate, CP7_E2alf, utilizes a companion E^{rns} ELISA. Animals vaccinated with CP7_E2alf are protected against

infection via the antibody response to the homologous E2 glycoprotein. The absence of CSFV E^{rns}, as determined by the companion E^{rns} ELISA, allows for differentiation between wildtype infected and vaccinated animals (Graham *et al.*, 2012; USDA APHIS, 2012).

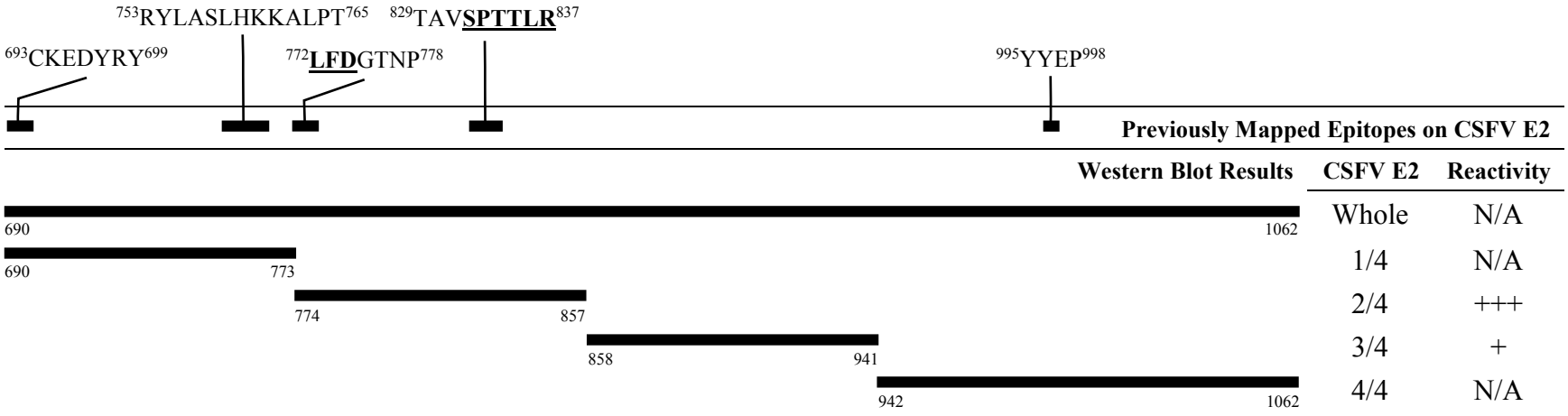
The functionality of CSFV E2 DIVA subunit vaccines are dependent on the ability of the companion ELISA to accurately detect the antibodies of interest. BVDV E^{rns} antibodies generated in response to BVDV infection in swine interfere with the ability to accurately differentiate infected from vaccinated animals. One study analyzed CSFV ELISAs for their use as a companion diagnostic assay. Of the two commercial assays they evaluated, one CSFV E^{rns} ELISA was completely unable to correctly identify CSFV E2 negative samples that contained other pestivirus E2 antibodies. The other ELISA they evaluated, although it was noted as being the improved assay, produced false-positive results 30% of the time (Schroeder *et al.*, 2012). This study further highlights the need for improved serological assays for the detection of CSFV as companion assays for DIVA vaccines.

The presence of BVDV antibodies in swine continue to be problematic for the surveillance and control of CSFV. The identification of cross-reactive and cross-neutralizing epitopes between CSFV and BVDV for the development of improved diagnostics and DIVA vaccines is necessary to account for this issue. The results of this study identified CSFV amino acid positions 774 through 857 and BVDV amino acid positions 783 through 872 as the regions that contain the epitopes recognized by cross-reactive antibodies between BVDV E2 and CSFV E2. These results provide more specific sequence regions to use for the improvement of CSFV diagnostic assays and DIVA vaccines.

Tables and Figures

Figure 4.1 Previously Mapped Epitopes of CSFV E2 from the Literature and Fragment Reactivity to Alphavirus CSFV E2 Polyclonal Swine Serum as Determined by Western Blot

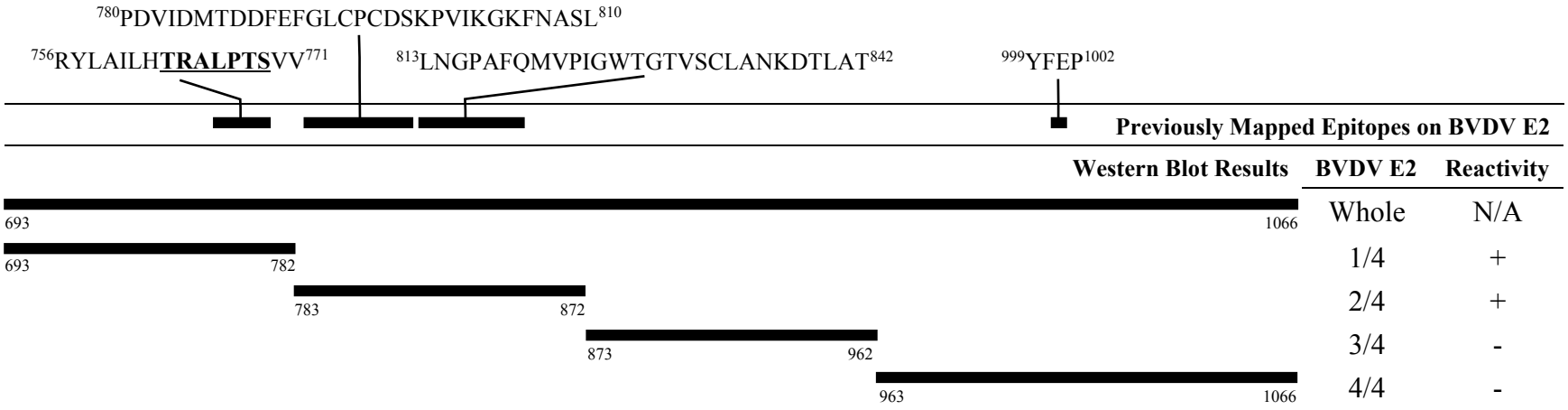
CSFV E2 amino acid positions are in reference to the whole polyprotein sequence of CSFV C-strain (GenBank accession number Z46258.1-1.) Epitope CKEDYRY was identified by Dong and Chen (2006). Epitope RYLASLHKKALPT was identified by Chang *et al.* (2012b). Epitope LFDGTNP was identified by Peng *et al.* (2008) and the LLFD motif was identified by Chang *et al.* (2010). Epitope TAVSPTTLR was identified by Lin *et al.* (2000) and the SPTTLR motif was identified by Zhang *et al.* (2006). Epitope YYEP was identified by Yu *et al.* (1996).



Western Blot Results	CSFV E2	Reactivity
	Whole	N/A
	1/4	N/A
	2/4	+++
	3/4	+
	4/4	N/A

Figure 4.2 Previously Mapped Epitopes of BVDV E2 from the Literature and Fragment Reactivity to Alphavirus BVDV E2 Polyclonal Swine Serum as Determined by Western Blot

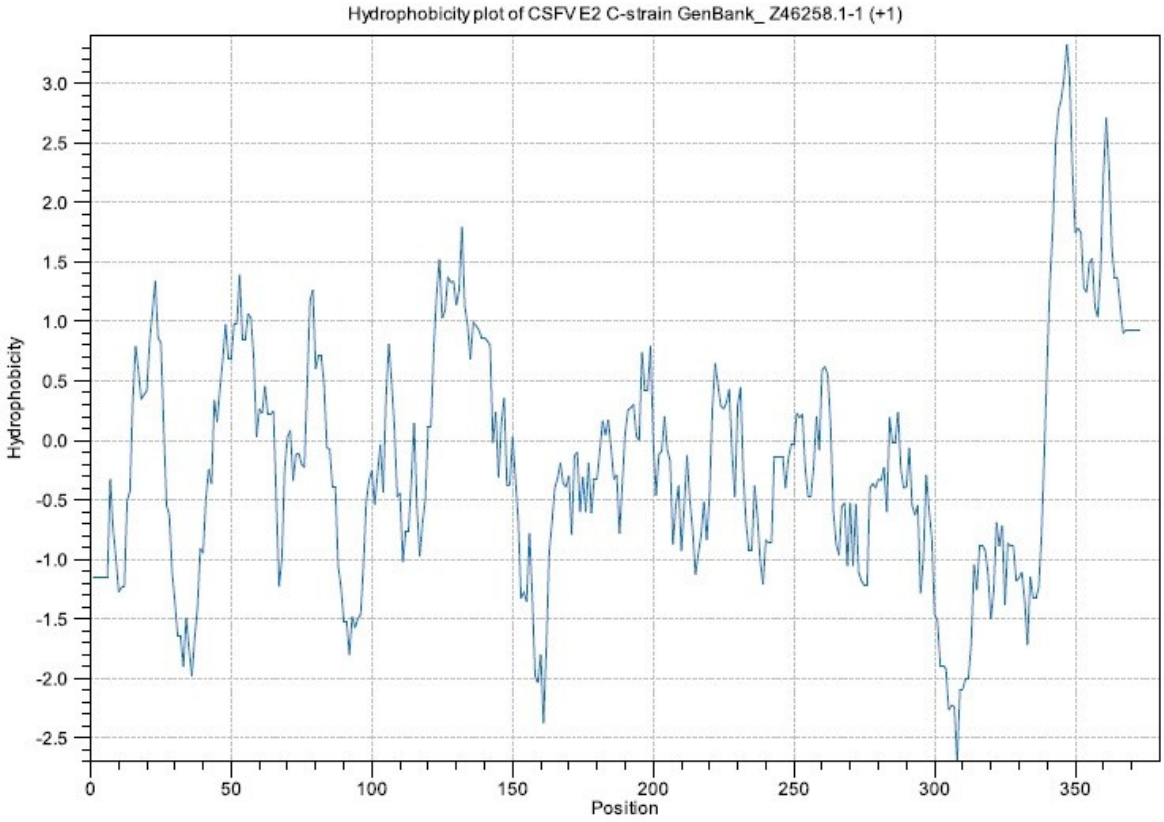
BVDV E2 amino acid positions are in reference to the whole polyprotein sequence of BVDV-1a Singer strain (GenBank accession number DQ0889) with the exception of epitope Y2 which is in reference to BVDV-2 strain C413 (GenBank accession number NC_002032.1). Epitope RYLAILHTRALPTSVV was identified by Paton *et al.* (1992b) and the motif TRAPLTS was identified by Deregt *et al.* (1998). Epitope Y2, or PDVIDMTDDFEFGLCPCDSKPVIKGKFNASL, was identified by Jelsma *et al.* (2013) and is specific to BVDV-2. Epitope Z2, or LNGPAFQMVCPIGWTGTVSCTLANKDTLAT, was identified by Jelsma *et al.* (2013). Epitope YFEP was identified by Yu *et al.* (1996).



Previously Mapped Epitopes on BVDV E2		
Western Blot Results	**BVDV E2**	**Reactivity**
693–1066	Whole	N/A
693–782	1/4	+
783–872	2/4	+
873–962	3/4	-
963–1066	4/4	-

Figure 4.3 Hydrophobicity Plot of CSFV E2

Hydrophobicity plot of CSFV C-strain E2 (Genbank accession number Z46258.1-1) generated using CLC Main Workbench. Values above 0.0 indicate areas of high hydrophobicity and values below 0.0 indicate low hydrophobicity. Amino acid positions 280-373 represent an area of high hydrophobicity.



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