

Diagnostic techniques for Classical Swine Fever virus

by

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D.V.M, Kansas State University, 2015

AN ABSTRACT OF A DISSERTATION

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Department of Diagnostic Medicine/Pathobiology  
College of Veterinary Medicine

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## Abstract

Classical swine fever virus (CSFV) is an enveloped, positive strand RNA virus, and member of the genus Pestivirus. It is a highly infectious and transmissible swine pathogen that threatens the global swine industry. The United States has been free of CSFV since 1977, however, monitoring the millions of domestic and feral pigs present in the US puts a significant strain on national surveillance efforts. There are no validated diagnostic techniques that can simultaneously sample multiple pigs (i.e. all pigs in a pen or barn). Similarly, there are no validated serological assays that can quickly test for CSFV without cross-reacting with other pestiviruses. The purpose of the first study was to establish a moderate CSFV-infectious model and determine how a single oral fluid sample from a pen of pigs can function as a diagnostic sample for detecting CSFV. Oral fluid (OF) and serum samples were collected from 10 pigs experimentally infected with CSFV Paderborn strain. Using RT-PCR, CSFV was detected in OF on 8 days post infection (dpi), and in the serum of one pig on 6 dpi. A single OF sample can, therefore, take the place of 10 serum samples to detect CSFV in a population. In a second study, monoclonal antibodies reactive to CSFV glycoproteins were generated in mice immunized with recombinant E2 and Erns antigens. Five E2-specific clones and two Erns-specific clones showed reactivity to CSFV-infected. Epitope mapping of the E2 clones showed that all reacted with the N-terminal portion of E2; a region highly variable among pestiviruses. Together with OF sampling, monoclonal antibodies can be used to develop new tools for improving CSF surveillance in large swine populations.

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## **Dedication**

I would like to dedicate this to my parents and grandparents who inspired and supported me my whole life. I also want to thank all my friends that supported me through two doctorates. I especially want to dedicate this to my mother, Nicoleta, and my wife, Jessica, and our soon-to-be daughter, Zoë. I love you all!

# **Chapter 1 - Overview of Classical Swine Fever Virus and challenges to surveillance**

## **Introduction and Brief History**

Classical swine fever (CSF) is recognized by the World Organization for Animal Health (OIE) as one of the most important swine diseases worldwide. The etiologic agent, classical swine fever virus (CSFV) is an enveloped, positive sense, RNA virus in the family *Flaviviridae*, and the Genus *Pestivirus*. *Flaviviridae* contains important human and animal viruses, such as, West Nile, Hepatitis C, and Zika (Lindenbach et al., 2007; Holbrook, 2017). The genetic organization and viral replication strategies of pestviruses is more similar to hepaciviruses (and pegiviruses) than with flaviviruses (Stapleton et al., 2011; Schweizer and Peterhans, 2014; Thézé et al., 2015). The *Pestivirus* genus contains only animal pathogens like bovine viral diarrhea virus (BVDV), border disease virus (BDV), and other more recently identified viruses like Bungowannah virus. The most important characteristics of all pestviruses are: 1) infect even-toed ungulates, 2) have the ability to cause acute disease, persistent infection, or asymptomatic infection, 3) possess unique protease and RNA nuclease genes that help in evasion of the host immune system, and 4) only the non-cytopathogenic biotype circulates in animal populations (Moennig and Plagemann, 1992; Schweizer and Peterhans, 2014). CSFV is further differentiated from other pestviruses since it only infects pigs and typically causes more severe disease than BVDV and BDV, which cause mild, persistent infections. Due to this more striking presentation, CSF was identified a century before the other pestviruses (Moennig and Plagemann, 1992; Moennig et al., 2003).

CSF was first described as hog cholera in the US in the early 1800s, but the disease quickly became endemic in both North America and Europe by the 1860s. From the United States, CSF spread to Canada and much of South America, although the exact timeline is uncertain (Edwards et al., 2000). By the early 1900s the disease was determined to be of viral etiology, with outbreaks extending throughout Asia, and Australia (“Emergency Animal Diseases Bulletin Protecting Australia from CSF | Australian Veterinary Association,” n.d.; Luo et al., 2014). Around this time, the first inactivated vaccines were developed, but offered limited protection. Then the advent of virus isolation and serial passage of highly virulent strains in surrogate animals (i.e. rabbits and gerbils) in the 1950s led to the production of modified live vaccines (MLVs), such as, the Chinese C-strain, the Japanese GPE-negative strain, and the French Thiverval strain. The lapinized C-strain vaccine was and still is the most widely used vaccine; it is still effective against the various CSFV strains throughout the world (J. T van Oirschot, 2003; Beer et al., 2007; Huang et al., 2014). Recent reports have shown that vaccination has applied selective pressure and driven evolution of CSFV in regions where vaccination is widespread (Chen et al., 2008; Tang et al., 2008; Leifer et al., 2012; Pérez et al., 2012; Ji et al., 2014). Furthermore, MLVs cannot be implemented in disease free regions, since it is impossible to differentiate vaccinated from infected animals (DIVA). More recently, DIVA-compatible subunit vaccines were developed; however, these vaccines do not offer the same quality of protection as MLVs (see Huang et al., 2014 for a recent review). Vaccination helped to bring the virus under control, but eradication was not achieved until strict stamping out policies were adopted.

Stamping out entails serologic testing of suspected cases and culling of all animals on the affected farm (and often neighboring farms depending on proximity). Strict quarantines and

regional movement restrictions are also employed to limit the spread of the disease. Australia implemented such policies each time CSFV was detected, and thus limited the disease to three, isolated outbreaks (“Emergency Animal Diseases Bulletin Protecting Australia from CSF | Australian Veterinary Association,” n.d.). The last Australian outbreak was stamped out in 1962 and the country has maintained its freedom from disease ever since (OIE WAHIS). Canada and the United States followed suit by adopting stamping-out policies, and have been disease free since 1963 and 1976, respectively (Walker, 1977; “OIE World Animal Health Information System,” n.d.). The European Union (EU) began to slowly employ stamping out tactics in the 1980s, and now CSF is mostly limited to eastern Europe (Postel et al., 2013a). The EU had the added difficulty of an endemically infected wild boar population (Elbers et al., 2000; Artois et al., 2002; Kaden et al., 2004; Simon et al., 2013; Goller et al., 2016). This along with the high population density of modern swine production systems, and movement of contaminated fomites contributed to several, costly outbreaks in domestic swine during the 1990s throughout the continent (Fritzemeier et al., 2000; Moennig et al., 2003). CSF in wild boar has been curtailed through successful oral bait vaccine campaigns (von Rügen et al., 2008; Moennig, 2015; Rossi et al., 2015). Mexico, Chile, Paraguay and Japan all used a combination of vaccination to reduce disease incidence, followed by stamping-out to fully eradicate CSF; today, they are all considered CSF-free (“OIE World Animal Health Information System,” n.d.). Several other countries have managed to eradicate the disease from regions with intensive pig farming, but still harbor CSFV in other regions (pink countries on Figure 1.1).

## **Transmission**

CSFV is highly transmissible, as it can be spread both horizontally and vertically; the latter can result in persistently infected (PI) animals as is seen with BVDV infection (Schweizer

and Peterhans, 2014). While transplacental infection has been shown in controlled studies for both domestic and wild pigs, its relevance in the field appears to be minimal (Depner et al., 1995; Kaden et al., 2005; Muñoz-González et al., 2015; Cabezón et al., 2017). Infection of young pigs is crucial for persistence of CSFV in wild boar, but there is no evidence that these pigs are typically infected *in utero* (Depner et al., 1995; Kern et al., 1999; Kaden et al., 2000, 2005). Horizontal transmission via the oronasal route is much more common, as the virus is viable in all body secretions. The virus is easily transmitted through a contaminated environment and fomites; if kept in cool place out of direct sunlight the virus remains viable on buildings for several days. CSFV remains viable in serum and tissues for days or even weeks if chilled; curing or smoking infected meat does not always inactivate the virus (Edwards, 2000; Cowan et al., 2015). Feeding pigs trash or swill contaminated with CSFV-infected products hampered early eradication efforts in North America and the European Union (EU), and is therefore illegal in many countries (Young and Walker, 1976; Fritzscheier et al., 2000). This highlights the need for rigorous hygiene and biosecurity protocols on farms and vehicles transporting swine (Edwards, 2000; Fritzscheier et al., 2000; Ribbens et al., 2007). Pigs presenting with acute disease secrete massive quantities of virus, but chronic disease can last months which leads to an even greater amount of excreted virus over the length of the infectious period. The infectivity of CSFV makes management of outbreaks very difficult (Van Oirschot et al., 1983; Terpstra and de Smit, 2000; Weesendorp et al., 2009a, 2009b). Furthermore, moderate chronic disease has such a wide variety of presentations that it's easy to confuse CSFV with other more common diseases like bacterial septicemia, highly pathogenic PRRSV, or PCV2-AD (Moennig et al., 2003). This delay in diagnosis proved especially costly for the massive CSFV outbreak in the Netherlands between 1997-98, and for a smaller outbreak in the UK (Stegeman et al., 2000; Paton, 2002).

## **Pathogenesis**

CSFV first establishes itself in the tonsillar epithelium, and then progresses to adjacent lymph nodes through the reticuloendothelial system. Due to its ability to efficiently infect endothelial cells and monocytes/macrophages CSFV causing acute disease can be detected in primary and secondary lymphatic organs by 2-3 days post infection (dpi). Around this same time gross lesions, and deteriorating clinical signs begin to develop as the virus replicates throughout the host (Ressang, 1973; Belák et al., 2008). Infection with moderately virulent strains spreads in the same fashion, but lesions and clinical signs appear more slowly (4-5 dpi), and with decreased intensity. As mentioned above, there is also increased variability in presentation amongst individuals as some will develop acute, fatal disease, while some develop a chronic form of the disease and remain largely asymptomatic until they succumb after several months (Cheville and Mengeling, 1969; Van Oirschot et al., 1983; Mittelholzer et al., 2000; Moennig et al., 2003; Belák et al., 2008). Additional clinical signs for acute presentations are conjunctivitis, poor attitude and poor body condition; more terminal disease signs may include neurological signs and skin hemorrhages. Again, these symptoms can be delayed in chronically infected pigs, and these pigs can also present with concomitant bacterial infections as the virus slowly depletes host leukocytes (Mittelholzer et al., 2000; Moennig et al., 2003; Blome et al., 2017).

## **Virulence**

The development of acute versus chronic disease depends heavily on the strain virulence, and infectious dose, but also host factors: age, and immune status (discussed below) (Petrov et al., 2014; Jenckel et al., 2017). Weesendorp et al. (2009) showed that increasing the inoculation dose of the moderately virulent Paderborn strain led to increased transmissibility, and more severe clinical disease. Both structural (Core, E1, E2, and Erns) and nonstructural proteins



(Npro, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) contribute to virus replication and its relative virulence. These proteins are encoded on a ~12.3 kb genome, contained on a single opening reading frame (ORF), which is flanked by two untranslated region (UTR) (Lindenbach et al., 2007; Schweizer and Peterhans, 2014). As summarized in Table 1.1, some of these proteins are more important for determining virulence, and have been extensively described in the literature (van Gennip et al., 2004; Tamura et al., 2012; Leifer et al., 2013).

For example, E2 is postulated to bind the CSFV-specific cell receptor, CD46, which initiates clathrin-mediated endocytosis (Dräger et al., 2015). E1, which forms heterodimers with E2, then mediates internalization (Weiland et al., 1990; Wensvoort et al., 1990; Wang et al., 2004; Fernandez-Sainz et al., 2009). These 2 proteins are essential for infection (Wang et al., 2004). E2 is also the most antigenic and immunogenic protein, which makes it the preferred target for diagnostic assays and vaccine development (Gavrilov et al., 2011). Along with the 5' UTR, it is a preferred target for RT-PCR assays for phylogenetic analysis (Greiser-Wilke et al., 1998; Björklund et al., 1999; Paton et al., 2000; Greiser-Wilke et al., 2007). E2 is almost always targeted by monoclonal antibodies (mAb) in standard immunoassays (i.e. virus isolation, IHC, virus neutralization) (Grieser-Wilke et al., 2007). Furthermore, as the main immunogen, neutralizing antibodies generated against E2 are fully protective. The N-terminal, B/C domain of E2 has been shown to contain several neutralizing epitopes that can be used as successful vaccines in subunit or epitope vaccines (van Rijn et al., 1992, 1993, 1994; Liu et al., 2006; Peng et al., 2008; Zhou et al., 2011; Liao et al., 2016). Multiple mutations in the structure or glycosylation of E2 can cause severe attenuation, which is why E2 is considered a major virulence determinant (G. R. Risatti et al., 2005a; Risatti et al., 2006, 2007a).

Erns possesses RNase activity and degrades excess viral nucleic acids to prevent stimulation of retinoic acid-inducible gene I (RIG-I) receptors, and toll like receptors (TLRs). These receptors are the first defense line against viral pathogens as they bind viral motifs (i.e. nucleic acids) and activate the innate immune response. By subverting them, Erns prevents upregulation of Type I IFN and blocks upregulation of NF- $\kappa$ B (L.-J. Chen et al., 2012). TLR3 has been identified as an important target of Erns activity of both CSFV and BVDV (Mätzener et al., 2009; Zürcher et al., 2014a, 2014b; Cao et al., 2015). Blocking RNase activity of Erns reconstituted normal production of IFN- $\alpha$  (Mätzener et al., 2009; Zürcher et al., 2014a). The impairment of TLR and pDC activation explains the lack of development of a sufficient immune response against CSFV. The Core protein has also been shown to affect the activation of inflammatory mediators, but these interactions have not been extensively characterized (Gladue et al., 2010, 2011; Li et al., 2014).

Similarly, Npro mediates the proteosomal degradation of the key intermediary molecule, interferon regulatory factor 3 (IRF3). This prevents production of Type I IFN in all cells except plasmacytoid dendritic cells (pDCs) (Ruggli et al., 2003; La Rocca et al., 2005; Bauhofer et al., 2007). These cells produce elevated amounts of IFN- $\alpha$  in response to CSFV infection (Summerfield et al., 2006; Jamin et al., 2008; Fiebach et al., 2011). While efficient IFN- $\alpha$  upregulation is observed in vaccinated animals (Balmelli et al., 2005), aberrant IFN- $\alpha$  production has been implicated as the main source of lymphoid depletion in primary and secondary lymphoid organs. This is the main contributor to the severe lymphopenia and leukopenia typically associated with CSFV infection (Susa et al., 1992; Summerfield et al., 1998, 2006; Jamin et al., 2008). Deletion of the entire Npro sequence attenuated CSFV *in vivo*, but Npro is also crucial for viral replication (Mayer et al., 2004). Knocking out only the immunomodulatory

function of Npro did not attenuate infection with a highly virulent strain, and only slightly attenuated infection with a moderately virulent strain. This indicates that Npro is more important for persistent and chronic disease, allowing the virus to replicate in different cell types for extended periods of time (Ruggli et al., 2009). Other nonstructural proteins, NS2, NS3 and NS4, have also been shown to contribute to virulence (Table 1.1).

### **Virus-Host Interactions and Immunity**

Despite the myriad of virulence genes, protection from CSFV can be achieved under natural infection and much more efficiently through vaccination. Both modified live (MLV) and subunit vaccines induce strong protection by stimulating production of neutralizing antibodies, primarily against the E2 glycoprotein (Terpstra and Wensvoort, 1988; J. T van Oirschot, 2003; Huang et al., 2014). As previously mentioned, subunit vaccines use E2 antigen, since it is the most immunogenic and most abundant CSFV surface protein. Because only one viral protein is expressed in vaccinated animals, they can easily be differentiated from infected animals which would express all viral proteins. This DIVA-compatibility is highly advantageous in emergency vaccinations for controlling outbreaks, and would be paramount in regions wishing to maintain a disease-free status. Unfortunately, clinical protection with subunit vaccines is only achievable 14 days post-vaccination, usually requires two separate immunizations and does not offer good cross-protection against multiple viral strains. This drastically limits their use in emergency vaccination situations. MLVs can induce protection as early as 3-5 days post-vaccination, but are not DIVA compatible and therefore cannot be used in disease-free regions (Huang et al., 2014; van Oirschot, 2003). The major goal facing CSFV vaccinology therefore, is to produce a vaccine that stimulates early protection, while being DIVA-compatible. The early protection is attributed to cellular immunity mediated by CD3<sup>+</sup>CD4<sup>-</sup>CD8a<sup>high</sup> T cells secreting IFN- $\gamma$  (Graham et al.,

2012b, 2012a, Franzoni et al., 2013a, 2013b). This protection provides sterilizing immunity and prevents the characteristic leukopenia brought on by CSFV infection. T-cell epitopes have been mapped to E2, NS3, NS4A and B, NS5A and B (Ceppi et al., 2005; Graham et al., 2012b)(Ceppi et al., 2005; Graham et al., 2012b).

It has also been shown that some pigs can successfully clear the virus with minimal clinical signs under natural infection (Depner et al., 1996; Hulst et al., 2012; Tarradas et al., 2014). To understand this phenomenon several studies have looked at the differential expression of host immune genes during CSFV infection. Using microarray and PCR techniques they saw that IFN regulatory pathways were consistently affected, with the more highly virulent strains inducing a stronger downregulation than more moderately virulent strains (Durand et al., 2009; Renson et al., 2010). Genes related to ubiquitination and the proteasome were also consistently upregulated. Together these findings point to Erns and Npro preventing IFN signaling or mediating the proteosomal degradation of key intermediates, respectively (Durand et al., 2009). Furthermore, these negative effects were noticed sooner (usually 1 DPI difference) with highly virulent strains. This is expected since pigs infected with more virulent strains show increased pyrexia and deterioration in clinical signs sooner than those infected with less virulent strains (Belák et al., 2008; Mittelholzer et al., 2000). Durand et al. (2009) also noted that genes related to the innate and adaptive immunity, and antigen presentation were differentially expressed, but no clear mechanisms could be established. Most recently, Hulst et al. (2012) showed that in contrast to protected pigs, chronically infected pigs do not upregulate any TLRs or other innate viral receptors that would stimulate a robust immune response. Understanding the factors responsible for host immunity is crucial for developing future CSFV vaccines and diagnostic assays.

## Genotype and Geographical Distribution

Today CSF persists in wild and domestic swine in Eastern Europe, Asia and South America. There are three main historic CSFV genotypes that are further subdivided into 11 subgroups (1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, 3.4) (Beer et al., 2015). Phylogenetic studies are critical for understanding the source and progression of different outbreaks, as well as the evolution of the CSFV over time and across populations of wild and domestic swine. Various groups have performed such analyses by sequencing one or more of the following genomic regions: 150 nt portion of 5'UTR, a 190 nt portion of E2, or a 409 nt portion of NS5B (Lowings et al., 1994; Greiser-Wilke et al., 1998; Björklund et al., 1999). Paton (2000) standardized the framework to integrate sequences from all three methodologies, allowing comparison and categorization of various isolates. Whole genomes can now be utilized to provide even finer analysis of existing and emerging isolates thanks to next generation sequencing, but this has only seen minimal use in CSFV research (Töpfer et al., 2013; Fahnøe et al., 2014). Table 1.2 outlines the most recent phylogenetic findings over the last 10-15 years.

Genotype 1, contains the longest circulating strains including “highly virulent” strains (e.g. Brescia and Koslov), as well as the MLV strains mentioned above. For much of its history CSFV caused acute, hemorrhagic fever causing high morbidity and mortality, but since the 1970s and 80s has transitioned to more moderate disease (Dunne, 1973). Today, subgroup 1.1 predominates throughout South America. It also circulates throughout Asia in both domestic and wild boar. Strains from subgroup 1.3 are limited to Central America (see Table 1.2). More recently, sequencing of the entire E2 glycoprotein revealed distinct genome changes in viruses isolated from Cuba, and thus led to the creation of subgroup 1.4 (Postel et al., 2012, 2013b). Viruses from subgroup 1.2 have not been recently isolated.

Genotype 2 (specifically 2.3) first appeared in Japan in the early 1970s (Paton, 2000). It gained prominence in the 1980s when it appeared in Europe with a milder disease presentation that was harder to identify with traditional CSFV disease (Paton, 2000; Mittelholzer et al., 2000). The Paderborn strain (subgroup 2.1), circulated undetected for weeks and was responsible for the \$2.3 billion outbreak in 1997-98 in the Netherlands (Meuwissen et al., 1999; Stegeman et al., 2000). This subgroup has since spread throughout Asia, but has receded to the eastern borders of the European continent. Subgroup 2.2 is confined exclusively to Asian swine, while subgroup 2.3 is the predominant European isolate in domestic and wild swine (Table 1.2). The situation throughout much of Africa is unknown, but the only significant outbreaks have been recorded in Madagascar. An outbreak did occur in South Africa in 2005, and was attributed to an isolate from subgroup 2.1 (Sandvik et al., 2005). Genotype 3 was also first reported in Japan in the 70's and then spread throughout Eastern and South-eastern Asia (Sakoda et al., 1999; Ozawa et al., 2006). This genotype has never been reported outside of Asia, and no recent isolates have been found in the last 15 years. Subgroup 3.4 was most recently implicated in Taiwan in 2002, but no outbreaks from that region have been reported in the literature or to the OIE since 2005 (Deng et al., 2005; Lin et al., 2007; "OIE World Animal Health Information System," n.d.).

## **Diagnostics**

Diagnostic assays for CSFV can be broken down into five main categories: clinical diagnosis, culture-based, tissue staining, serological, and nucleic acid based methods (Table 1.3). All tests protocols must be validated by the OIE for results to be considered for official surveillance programs; the approved protocols are contained in the OIE Terrestrial manual (2014). Clinical diagnosis relies on observation of the lesions described in the Pathogenesis section and the experience of the producer or veterinarian observing them. This method is the

most unreliable, but a majority of CSFV cases in German outbreaks in the 1990s were diagnosed based off clinical signs (Fritzemeierj et al., 1999). Engel et al. (2005) calculated that veterinarians diagnosed CSFV infected herds with a sensitivity of 90% only after 47 days had passed since initial virus introduction on the premise. Even during the 1997-1998 outbreak, Dutch veterinarians had trouble differentiating CSFV from other, similar diseases (Stegeman et al., 2000; Elbers et al., 2002, 2003). In areas where veterinarians are not expecting to see the disease the diagnosis could be substantially delayed if clinical observations are the main diagnostic tool.

The culture-based immunoassays are the oldest laboratory techniques for detecting CSFV and rely on growing the virus on either swine primary cells (pulmonary alveolar macrophages, peripheral blood mononuclear cells), or immortalized cell lines (swine kidney-6 cells or porcine kidney-15 cells) (Grummer et al., 2006). While all these tests are relatively sensitive, their main drawback is that one must wait 3-4 days for the virus to grow on cells. This is much slower than the newer assays discussed below. Furthermore, the cell type used can differ from one diagnostic lab to the next and thereby affect reproducibility of results. Virus isolation (VI) was the main confirmatory test in the past, and relies on fluorescent or horseradish peroxidase (HRF)-conjugated antibodies for visualization. Depending on the antibodies used, cross-reactivity with other pestiviruses must also be considered. VI entails culturing blood, or tissue homogenates from infected pigs on CSFV-permissive cells; this is followed by fixation and staining with antibodies. Virus neutralization (VN) assays are still considered the gold standard and have very good sensitivity; however, sensitivity suffers if the virus strain used to produce the VN is not the same as the infectious strain being tested (Blome et al., 2006; OIE Terrestrial Manual, 2014). These assays also have problems detecting antibodies if performed too early or too late after

initial infection; additionally, not all pigs produce neutralizing antibodies. Neutralization assays can be read using either immunofluorescence assays (IFA) or neutralizing peroxidase-linked assay (NPLA); the latter is now preferred by the OIE since it is easier to interpret results and does not require a fluorescent microscope (Terpstra et al., 1984; OIE Terrestrial Manual, 2014). The main difference between the two is that the secondary antibody used to detect the virus is conjugated to either a fluorescent probe (IFA) or HRP (NPLA).

Tissue staining methods include Immunohistochemistry (IHC) and the immunofluorescence antibody test (IFAT). IHC detects viral antigens from formalin-fixed and paraffin-embedded tissues collected at necropsy; it was the main method of studying CSFV tissue distribution in the past (Cheville and Mengeling 1969; Ressang, 1973). Today, it can be complimented by *in situ* hybridization (which detects viral RNA in fixed tissues), and it is still used to validate other detection methods (e.g. PCR) in tissues (Liu et al., 2011; Zhang et al., 2017). IHC is very labor intensive, usually requiring a specialized diagnostic lab, and its sensitivity is contingent upon the inspecting veterinarian collecting appropriate tissue samples. For this reason, it is a poor choice for ruling out disease in a herd. IFAT utilizes cryostat (frozen) tissues instead of formalin fixed and paraffin embedded tissues like IHC. Therefore, the former utilizes a quicker fixation and processing step than IHC. However, IFAT still requires highly specialized equipment and expertise, and it requires that several symptomatic animals be tested due to its relatively low sensitivity (Bouma et al., 2001; OIE Terrestrial manual).

Serological methods are very quick and easy to perform, and equally easy to automate or perform with limited equipment in the field. These assays utilize monoclonal antibodies (mAbs) to detect viral antigens or antibodies generated against the virus. Unfortunately, due to the similarity of Pestivirus antigens, these monoclonal antibodies are subject to cross-reactivity and



therefore limited specificity (de Smit et al., 2000; Dewulf et al., 2004). The quickest and easiest antibody-based technique is the enzyme-linked immunosorbent assay (ELISA); direct, indirect, competitive and complex trapping-blocking (CTB) ELISAs are available (OIE Terrestrial Manual, 2014). CTB ELISA are the most specific ELISAs and are recommended by the OIE. This method utilizes two complimentary mAbs that react to separate epitopes on the same antigen (usually E2). One mAb is coated onto the ELISA plate and another is conjugated to HRP. The reactive antigen is incubated with test sera and added to the plate with the primary mAb. Then the secondary mAb is added; if both antibodies react the HRP induces a visible, chromogenic reaction, but if antibody from the test sera reacts with the antigen it blocks one of the monoclonal antibodies and no reaction is seen. Therefore, antibody titer in the test sera is inversely proportional to the chromogenic reaction (Wensvoort et al., 1988). Unfortunately, even the CTB ELISA can cross-react with other Pestivirus samples, making them unreliable for use in surveillance in disease-free regions (de Smit et al., 2000). To improve the DIVA capability of E2-subunit vaccines, complementary ELISAs that screen for Erns have been developed (Floegel-Niesmann, 2001; Aebischer et al., 2013; Meyer et al., 2017). These have the capability to distinguish vaccinated pigs (which only express E2) from infected pigs (which express both E2 and Erns); however, cross-reactivity with other pestiviruses was still observed. A new, alternative serological assay is fluorescent microsphere immunoassay (FMIA). In principle FMIA works the same as ELISA, but the target antigens or antibodies are coupled to the fluorescent beads instead of a plate. The bound beads are then incubated with samples and then a biotinylated detection antibody is added. This secondary antibody can be stained with streptavidin and detected on a specialized flow cytometer or specialized plate reader, which correlate fluorophore activation with binding of the target. The advantage of FMIA is that

multiple antigens or antibodies can be bound to the beads, allowing for detection of hundreds of targets in single reaction (Krishhan et al., 2009). This means that CSFV surveillance could be easily integrated into surveillance for more common pathogens (Deregt et al., 2006; LeBlanc et al., 2009; Xia et al., 2015). However, these assays must be run on specialized machines and require significant expertise to set up. Further validation is required before these assays can be confidently recommended for OIE or national surveillance efforts.

Reverse transcription polymerase chain reaction (RT-PCR) is a widely used screening procedure for CSFV. Today's PCR protocols are considered more sensitive than culture based methods, and are also much faster and easier to use (Dewulf et al., 2005; Greiser-Wilke et al., 2007). The older gel-based methods for PCR have now been replaced by real-time protocols: these are faster, more sensitive, and can quantify virus levels in blood and tissue samples (Hoffmann et al., 2005; G. Risatti et al., 2005; Le Dimna et al., 2008; Dias et al., 2014). RT-PCR primer/probes usually target a conserved region in the 5' UTR. Due to the specificity accorded by custom PCR primers and TaqMan probes, this technique can also be used to differentiate different strains. Through multiplexing, this can even be done in a single reaction (Huang et al., 2009; Leifer et al., 2009). A disadvantage of PCR is the potential for degradation of RNA if samples are not properly stored and preserved. A trade-off for specificity and sensitivity is the lack of pen-side kits that would facilitate diagnosis in the field. Recent advancements in reverse transcription loop-mediated isothermal amplification (RT-LAMP) could address these issues. These protocols are even faster than PCR, and utilize a constant temperature, eliminating the need for energy-demanding thermocyclers. Furthermore, positive results can be judged by the naked eye through changes in color or turbidity (Yin et al., 2010; Zhang et al., 2010; Postel et al., 2015). RT-LAMP protocols still need to be validated by the OIE before they can be used in

official surveillance efforts. RT-PCR and RT-LAMP assays have also been designed to differentiate vaccinated from infected animals, and have shown efficacy even differentiating MLVs from wild-type virus (Leifer et al., 2009; Zhang et al., 2010). Innovations in diagnostic assays, such as, FMIA and RT-LAMP are crucial in improving surveillance for CSFV and other foreign animal diseases (FADs).

### **Limits to Surveillance**

There are many foreign animal diseases that threaten the US swine industry. Yadav et al. (2016) showed through computer modeling, that in the event of outbreaks with either FMD or CSF, the latter is likely to persist for an extended time before being eradicated. Additionally, it can cause both severe acute disease and chronic, persistent disease, making definitive diagnosis difficult (Mittelholzer et al., 2000; Stegeman et al., 2000). This makes CSFV an ideal model to assess the preparedness of US animal health agencies and its surveillance capabilities. Ideally, the CSFV surveillance program would detect and eliminate a potential infectious source before it ever entered the country. However, as the recent porcine epidemic diarrhea virus outbreak showed, viruses can often slip past our safeguards (Huang et al., 2013). PEDV is believed to have spread from China to the US in contaminated feed; it is not endemic throughout much of the country, and has extended into Canada and Mexico. This outbreak caused the loss of ~10% of US swine and extensive economic damage. Fortunately, this outbreak did not lead to significant trade restriction, as it is endemic in much of Europe and Asia (Lee, 2015). If this had been CSFV the loss of animal life and trade would have been significantly greater. Paarlberg et al. (2009) calculated that re-introduction of CSFV into the US would cost over \$4 billion, with full recovery occurring 4 years after outbreak detection. Effective surveillance for foreign animal

diseases like PEDV and CSFV, rely on international cooperation to assess risk in endemic region in an effort to prevent their spread to uninfected countries.

The OIE provides a great service by tracking and reporting disease events for the world's most important animal diseases. Their guidelines and regulation provide an important framework by which countries can implement their own disease management programs. However, the biggest limitation of any international organization like the OIE, is that it relies on participation from the individual member states to generate information on various diseases. Many countries provide up-to-date reports on a regular basis, but for others there can be large gaps in reporting; others still (i.e. Honduras and Mongolia) are not represented at all. China for example has not given an official report on CSFV since 2015. This is not to criticize how any country chooses to allocate its funds in protecting its animal populations, nor is it criticizing the efforts of the OIE. Different countries have different surveillance strategies and capabilities, and the OIE has the daunting task of somehow integrating them all. However, when assessing the risks to US agriculture, it is important to remember that these lapses in surveillance exist, often in the most vulnerable areas. Ensuring that CSFV remains outside the country begins not by pointing fingers, but by bolstering collaborative research efforts in regions where the virus is endemic, as these areas pose the greatest risk to US agriculture. If the virus population in these areas changes it can often have global ramifications.

Surveillance at the national level is also imperfect: APHIS admits that the current reporting system covers a much more limited population than the surveillance plan aims to cover. Small swine operations typically do not have a working relationship with a local veterinarian. This is problematic since local practitioners initiate a majority of reports, and these smaller operations usually practice less stringent biosecurity measures than large, well-managed

swine productions. Active surveillance involves sampling usually very few feral swine, and condemned carcasses at slaughter plants. In the event of an outbreak the virus could spread to many animals by the time these methods would detect it (“USDA APHIS Classical Swine Fever Response Plan: The Red Book,” 2013). Since producers and local veterinarians are the first line of defense, APHIS must expand its education campaigns to ensure that these individuals can identify signs of CSFV and report it to the appropriate authorities.

Unfortunately, even the best surveillance measures can fail to prevent disease incursion. For example, Crauwels et al. (1999) calculated that even if monthly serological surveillance was carried out for all Dutch swine herds, the probability of detecting the 1997 CSFV outbreak within 40 days after initial infection was still below 40%. This sampling scheme would be very difficult to implement with current sampling methods, especially in the US, which has 10 times more pigs than the Netherlands (“OIE World Animal Health Information System,” n.d.). As already mentioned, CSFV can survive for extended periods of time in various pig products (Edwards, 2000; Cowan et al., 2015). Therefore, it could easily be brought into the country via any sort of commercial transit. US customs agents confiscate tons of illegal (and potentially contaminated) animal products at various ports of entry each year (Bair-Brake et al., 2014; “Agriculture Fact Sheet | U.S. Customs and Border Protection,” n.d.). The situation is similar in Europe, where many of the confiscated animal products were found to carry foodborne bacterial pathogens (Schoder et al., 2015; Jansen et al., 2016). Despite the best efforts of border officers, tons of illegal animal products still make it into the US and are sold on the black market (News, 2007). Once inside the country, the infected material still needs to make its way to susceptible pigs. While most large-scale pig farms typically have sufficient biosecurity to avoid this scenario, the infected material has a much better chance of coming into contact with feral swine.

While these animals have been in US for centuries, their population has skyrocketed in the last decade. It is now estimated at approximately 5 million individuals, and their range has been continuously expanding (APHIS brochure). Furthermore, feral swine frequently come within 100 meters of domestic swine operations, which greatly facilitates disease spread (Wyckoff et al., 2009).

The preceding scenario assumes that CSFV infection of domestic swine would be accidental; however, it could also be done intentionally to cripple US agriculture. Both foreign, religious extremists and domestic, animal right's extremists (i.e. ALF) could commit and have committed agroterrorism (Keremidis et al., 2013). Terrorists could acquire and transport infected pig products much more easily than weapons or explosives. While the damage to human lives would be less severe, the economic damage could come out to billions of dollars (Casagrande, 2002; Wheelis et al., 2002). Eradication efforts could also cause the culling of hundreds of thousands of animals and psychological trauma on affected farmers (Terpstra and de Smit, 2000; Cohen et al., 2007). Fortunately, there is a lot of cross-application between defense against agroterrorism and foreign animal diseases, since strict biosecurity and rigorous surveillance are the main weapons for both. The best way to improve preparedness for an agroterrorist attack is to increase coordination between state and federal law enforcement with state and local animal health agencies. Earlier this year the US passed the "Securing our Agriculture and Food Act", which did just that: the Department of Homeland Security is now directed to provide oversight over defense of the country's food, agriculture, and veterinary systems (Young, 2017).

The US and APHIS must continually reassess the best possible response plans in the event of a CSFV outbreak based on new research. While stamping-out has successfully eradicated CSFV in multiple countries (including the US), its ethicality has been called into

question. Massive depopulation campaigns lead to culling of many uninfected animals, and have severe psychological impacts on farmers and erode confidence in central authorities (Cohen et al., 2007). Several have suggested the possibility of using emergency vaccination to get outbreaks under control before culling, which would limit the number of animals euthanized (J. T. van Oirschot, 2003; Boklund et al., 2009; Mourits et al., 2010). The APHIS Preparedness plan for CSFV does not exclude the use of emergency vaccination in the event of a US outbreak. Additional steps should be taken to outline the criteria for when this would be appropriate. Specific vaccines should also be developed for ramp-up production in the event of an outbreak. Furthermore, state and federal authorities should stage training exercises on how this sort of vaccination would be best implemented. Future research efforts should also focus on developing diagnostic techniques that sample multiple animals while also screening for multiple pathogens.

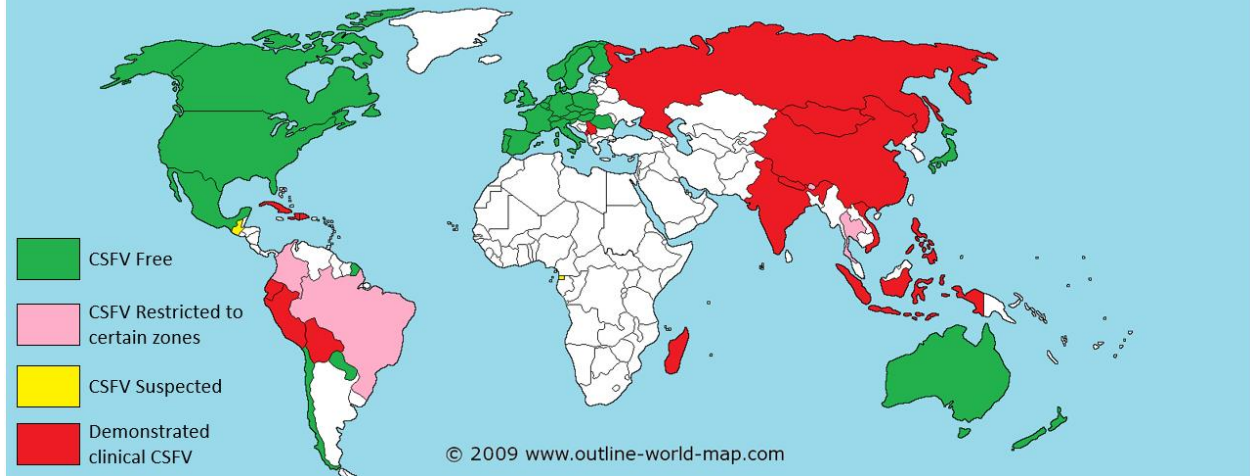
## **Conclusion**

While the number of global CSFV outbreaks has decreased over time, it is still present in many parts of the developing world. Not only does it cause extensive harm to the swine industry in these regions, but its continued persistence and evolution increases the risk of its reintroduction into disease-free areas. Furthermore, new challenges from other emerging diseases like African Swine Fever can shift focus away from CSFV control policies and allow the latter to recapture lost territory. Humanitarian crises brought on by war or natural disasters can also work to spread CSFV and many other diseases. International cooperation therefore becomes even more crucial for tracking and controlling the virus in vulnerable areas. Meanwhile, researchers should continue to develop new and effective diagnostic assays and DIVA-compatible vaccines. Surveillance efforts are critical for limiting the damage of CSFV in both endemic and disease-free regions, but face some significant limitations. First, due to the millions of pigs present in the

major swine producing countries (i.e. US, China and EU) and the thousands of pigs present on a single farm, sampling all these animals is impossible via blood sampling. Therefore, we must develop techniques that can sample entire pig pens more quickly and efficiently. Second, serological assays are quick and easy to use in the field, but are subject to cross-reactivity, which makes them unreliable for routine surveillance. New assays should be developed that are more specific, ideally these could be used as multiplex assays to detect both common and foreign animal diseases simultaneously.



## Figure 1.1 Worldwide CSFV Status 2017



**Figure 1.1 Worldwide CSFV Status**

Data compiled from OIE WAHIS databases. Countries in white are not officially CSFV-free, but had not reported CSFV to the OIE in the last reporting period, or no data was available.

Countries demonstrating clinical disease (red) – Ecuador, Cuba, Haiti, Dominican Republic, Bolivia, Peru, Madagascar, Serbia-Montenegro, Russia, Mongolia, China, India, Nepal, Vietnam, Indonesia, Timor-Leste, Cambodia, Philipines. Countries where CSFV is restricted to certain administrative zones (pink) – Bhutan, Colombia, Thailand, Brazil. Countries where CSFV is suspected but not confirmed (yellow) – Guatemala, Equatorial Guinea.

<b><u>Gene</u></b>	<b><u>Contribution to Viral Replication</u></b>	<b><u>Contribution to Virulence</u></b>	<b><u>References</u></b>
<i>5' UTR</i>	Initiates cap-independent translation, houses internal ribosome entry site (IRES).		Thiel et al., 1991; Fletcher and Jackson, 2002
Npro	Protease unique to pestiviruses. Initiates cleavage of the polyprotein.	Mediates proteolytic degradation of Interferon regulatory factors, preventing IFN upregulation. Deleting protease function only attenuates moderately virulent strains.	Bauhofer et al., 2007; Ruggli et al., 2009; Tamura et al., 2014
<b>Core</b>	Interacts with viral RNA and membrane to form nucleocapsid structure.	SUMOylation of Core prevents viral clearance and enhance proliferation.	Lindenbach et al., 2007; Li et al., 2014; Gladue et al., 2010 and 2011
	Can interact with the NS5B polymerase to upregulate RNA synthesis.	Interaction with hemoglobin beta influences IFN signaling, CSFV works to suppress hemoglobin beta for this reason.	
	Has been shown to enhance transcription.	Binds IQGAP1 (cytoskeleton regulator); the efficiency of this binding impacts viral replication and inhibits immune cell migration.	
<b>Erns</b>	Initial attachment to heparan sulfates.	RNase activity degrades viral RNA to prevent RIG-I and TLR-mediated activation of type I IFN.	Meyers et al., 1999; Sainz et al., 2008; Dräger et al., 2015
		Altering N-glycosylation sites causes attenuation.	
<b>E1</b>	Forms heterodimer with E2; necessary for internalization of virus into cells.	Altering N-glycosylation sites causes attenuation.	Wang et al., 2004; G. R. Risatti et al., 2005b Wang et al., 2004; Fernandez-Sainz et al, 2009; Dräger et al., 2015;
<b>E2</b>	Binding CD46 to initiate clathrin-mediated endocytosis.	Altering E2 fusion peptide or tertiary structure causes attenuation.	G. R. Risatti et al., 2005a; Risatti et al., 2006, 2007a, 2007b; Wang et al., 2004; Dräger et al., 2015; Holinka et al., 2016
		Immunization with E2 from BVDV confers protection. Best antigen used in subunit vaccines; anti-E2 antibodies are sufficient for protection against infection.	
		Altering O- or N-glycosylation sites causes attenuation.	

P7	Forms viroporin ion channel. Necessary for infectious virus, but not RNA replication.	Mutations in p7 can severely attenuate viral growth <i>in vivo</i> .	Gladue et al., 2012
NS2	Together with host cofactor Jiv90, generates autoproteolytic cleavage of NS2-3 precursor; this is necessary to activate serine protease function of mature NS3.	Dysregulation of NS2-3 cleavage attenuates viral growth.	Moulin et al., 2007; Tang et al., 2010, 2011; Lamp et al., 2011
		Cell cycle arrest in S-phase, which promotes viral replication.	
		NS2 interferes with inflammatory mediators to help establish persistence.	
NS3	Serine protease, NTPase, RNA helicase domains work synergistically to regulate cleavage of downstream products, which constitute the viral replication complex.	Excessive accumulation has been shown to increase cytopathic effect, which attenuates the virus <i>in vivo</i> .	Moulin et al., 2007; Gallei et al., 2008; Xiao et al., 2008; Zhu et al., 2010; Lamp et al., 2011
		Helicase activity promotes IRES-mediated translation.	
NS4A	Cofactor for serine protease function of NS3.	Influences RNA replication and virion formation.	Moulin et al., 2007;
NS4B	Essential to the viral RNA replication complex.	Acts w/ E2 to increase virulence. Cleavage of its proposed TIR-domain severely attenuates.	Fernandez-Sainz et al., 2010; Tamura et al., 2012
		NS4B affects activation of inflammatory mediators (i.e. TLR7, IL-6).	
NS5A	Essential for the viral RNA replication complex.	Can induce cell autophagy which is good for CSFV maturity, but also immune evasion.	(Zhu et al., 2010; Y. Chen et al., 2012; Sheng et al., 2012a, 2012b, 2014)
	At low concentration binds NS5B and increases replication. At high concentrations binds 3'UTR and inhibits RNA replication.		
NS5B	RNA dependent RNA polymerase.	Both NS5A and NS5B can inhibit NS3 complex formation to regulate translation.	Xiao et al., 2004; Zhu et al., 2010; Sheng et al., 2012b
	Binds 3'UTR to initiate RNA replication.		
3' UTR	Initiates RNA replication.		Thiel et al., 1991; Björklund et al., 1998

**Table 1.2 CSFV Genotypes described in the past 10-15 years**

	Asia	Americas	Europe	References
<b>Genotype 1</b>				
1.1	China, India, <u>Russia</u>	Brazil, Colombia, Peru, Ecuador, Bolivia		Vlasova et al., 2003; Pereda et al., 2005; Sabogal et al., 2006; Araínga et al., 2010; Patil et al., 2010; Jiang et al., 2013; Luo et al., 2014; Roychoudhury et al., 2014
1.3		Honduras, Guatemala		Pereda et al., 2005
1.4		Cuba		Postel et al., 2013b
<b>Genotype 2</b>				
2.1	China, Israel, South Korea, Mongolia, Taiwan, Thailand		Lithuania	David et al., 2011; Jiang et al., 2013; Postel et al., 2013a; Luo et al., 2014; Kwon et al., 2015; Luo et al., 2016; Enkhbold et al., 2017
2.2	<u>India</u> , China, Mongolia			Patil et al., 2010; Luo et al., 2014; Roychoudhury et al., 2014; Bhaskar et al., 2015; Barman et al., 2016; Enkhbold et al., 2017
2.3			Romania, Bulgaria, Croatia, Latvia, Bosnia & Herzegovina, Albania, Croatia, Macedonia, Serbia; Germany*, France*, <u>Slovakia</u> , Hungary*	Blome et al., 2010; Postel et al., 2013a
* – Indicates isolation from wild swine samples only <u>Underline</u> – indicates isolation from both domestic and wild swine				

<b>Table 1.3 Diagnostic Assays for Classical Swine Fever</b>		
<b><u>Test</u></b>	<b><u>Advantages</u></b>	<b><u>Disadvantages</u></b>
<u>Clinical diagnosis</u>	Cheapest and most common for initial diagnosis.	Unreliable even with experienced clinicians, always requires confirmatory tests.
<b>Culture based</b>		
<u>Virus Neutralization</u>	Gold standard for sensitivity and specificity.	Time consuming, hard to reproduce results. May require specialized microscope.
<u>Virus Isolation</u>	Very sensitive, indicates active infection.	Time consuming, hard to reproduce results. May require specialized microscope.
<b>Tissue staining</b>		
<u>IHC</u>	Direct visualization of viral antigens in tissue.	Requires specialized equipment, expertise, and confirmatory test.
<u>IFAT</u>	Quick and direct visualization of antigens in tissue during active outbreak.	Requires specialized equipment, expertise, and confirmatory test.
<b>Serological</b>		
<u>ELISA</u>	Fastest assay, does not require specialized equipment.	High potential for cross-reactivity with other Pestiviruses even with blocking assays.
<u>FMIA</u>	Can be performed quickly. Potential for multiplexing with other pathogens	Requires specialized equipment and expertise.
<b>Nucleic Acid Based</b>		
<u>RT-PCR</u>	Fast, sensitive, and specific.	Specialized equipment. Possibility for false results due to sample degradation.
<u>RT-LAMP</u>	Faster than PCR, only needs one stable temperature.	Requires expertise for primer design; not yet validated by OIE

## Chapter 2 - Oral fluid diagnosis for Classical Swine Fever virus

### Introduction

Classical Swine Fever virus (CSFV) is a positive sense, single-stranded RNA virus (12.5 kb) in the genus *Pestivirus*, family *Flaviviridae* (Lindenbach et al., 2007; Schweizer and Peterhans, 2014). The CSFV genome is completely contained on a single open reading frame (ORF) and encodes 4 structural genes (E1, E2, Erns and Core protein) and 7 non-structural genes (Npro, NS2, NS3, NS4A, NS4B, NS5A, NS5B). Both structural and nonstructural proteins are involved in virus replication and all contribute to virulence to some degree (Lindenbach et al., 2007; Leifer et al., 2013). The ORF is flanked on either side by untranslated regions (UTRs) that are involved in virus replication and translation of viral proteins (Thiel et al., 1991; Björklund et al., 1998; Fletcher and Jackson, 2002). The virus is currently endemic throughout most of Asia, as well as parts of Central and South America (Beer et al., 2015; Blome et al., 2017). CSFV disease manifests in three main categories: acute fatal disease, chronic disease and eventual death, and recovery. When studying this virus, it is important to establish an infectious model that permits observation of all three presentations. All three presentations can be seen during a single outbreak, making it difficult to differentiate CSFV from other diseases, such as, PRRSV, PCV-AD, or bacterial septicemia (Moennig et al., 2003).

Misidentifying CSFV infections is especially problematic in areas free from CSFV as it allows the virus to spread and makes outbreak management very difficult. This was evident with the outbreak in 1997-1998 in the Netherlands, which spread unnoticed for 5-7 weeks despite visits by veterinarians to affected herds and post-mortem examinations of CSFV-infected pigs (Stegeman et al., 2000). Furthermore, to maintain their status CSFV-free regions do not

vaccinate their swine herds, but rely on strict biosecurity and stamping out policies (culling all animals on infected premises and contact premises) (Terpstra and de Smit, 2000). Therefore, if a CSFV outbreak is detected in these regions it can completely shut down the swine industry. The outbreak in the Netherlands cost \$2.3 billion dollars to eradicate, and millions of healthy animals had to be pre-emptively culled to contain disease spreading (Meuwissen et al., 1999; Stegeman et al., 2000; Terpstra and de Smit, 2000). Like the Netherlands, the US has a rigid stamping-out policy; in the event of an outbreak large quarantine regions would have to be established and thousands, if not millions, of pigs would be culled to limit the spread of CSFV. A large-scale outbreak would seriously jeopardize the \$23.4 billion swine production contributes to GDP (“USDA - NASS, Census of Agriculture,” 2012). Furthermore, the US accounts for a third of the global swine export market, so trade restrictions on the US could have global ramifications (“Top 10 Pork-Producing Countries,” 2015).

The Animal and Plant Health Inspection Service (APHIS) is responsible for keeping the US free of foreign animal diseases like CSFV (“USDA APHIS Classical Swine Fever Response Plan: The Red Book,” 2013). Currently the APHIS surveillance plan relies for CSFV on swine producers and local veterinarians to report suspected cases. Depending on risk assessment criteria the agency will require certain states to conduct minimal active surveillance in feral swine and in slaughter plants deemed to be “high risk”. However, the current surveillance plan does not cover enough pigs, and in the event of an outbreak the ability of APHIS to track the virus in populations is limited to serum sampling (“Appendix B: Classical Swine Fever (CSF) Surveillance Plan,” 2007). Because this relies on testing individual animals it makes it difficult to get an accurate sample of the entire population on large pig farms. Serum sampling must be replaced with a technique that can screen entire pig farms more efficiently and cheaper.

Oral fluid (OF) sampling is a great alternative for surveillance, that has yet to be validated for CSFV diagnosis. OF consists of saliva (produced by salivary glands) and gingival crevicular fluid (secreted from the gums) (Delima and Van Dyke, 2003; Llana-Puy, 2006). The latter contains antibodies and antigens just like serum, which can be used for diagnosing various pathogens (Prickett and Zimmerman, 2010). In humans, OF are a well-established diagnostic sample for viruses such as, HIV and hepatitis C virus (Gallo et al., 1997; Cameron and Carman, 2005). In pigs, OF sampling is done with cotton ropes which are chewed on by multiple pigs in a pen. Studies have shown that this type of OF sampling is effective at diagnosing viruses such as, PRRSV, PCV2, and SIV (Prickett et al., 2008, 2011; Kittawornrat et al., 2010; Detmer et al., 2011). It minimizes handling stress on animals, does not require specialized training or equipment to collect or store, and is much cheaper than serum sampling. Ramirez et al. (2012) showed that rope sampling can be implemented by farm workers and used to track multiple viruses on different farms for 18 weeks at a fraction of the cost of traditional serum sampling.

OF studies on CSFV have been done in individual pigs (Grau et al., 2015; Petrini et al., 2017), and in populations of wild and domestic pigs with high and low virulence isolates (Mouchantat et al., 2014; Dietze et al., 2017). However, this study focuses on the effectiveness of oral fluids from ropes as a pooled population sample under the different disease presentations of moderately virulent infection. Unlike the latter two studies, we also ensured that the ropes we used were kept off the ground to limit environmental contamination from feces and urine. The objective of this study was to show proof of concept that OF could be collected and used to diagnose CSFV in a group of experimentally infected pigs. We also wanted to create an infectious model that would induce different disease states, as would be encountered in the field. To this end, we utilized the moderately virulent Paderborn strain, that was isolated from the



1997-98 outbreak in the Netherlands (Widjoatmodjo et al., 1999). The results presented are an important first step in validating OF sampling as an improvement on current serum surveillance methods.

## **Methods**

### Ethical statement

Experiments involving animals and virus were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

### Virus and Cell culture

The CSFV Paderborn strain was propagated by single passage on porcine kidney 15 (PK15) cells. The PK15 cells were maintained in MEM culture media (Life Technologies) supplemented with 7% fetal bovine serum, L-glutamine, 0.01% Penicillin/Streptomycin, and 0.008% Amphotericin-B (Gibco) in a 37°C and 5% CO<sub>2</sub> incubator. Virus was quantified by end-point titration performed in triplicate on PK15 cells seeded on a 24-well plate (Falcon). Infected cells were visualized by indirect immunofluorescence assay (described below). Log<sub>10</sub> tissue culture infectious dose 50 (TCID<sub>50</sub>) was calculated using the Reed-Muench method (1938).

### Animals

We used 10 White x Landrace pigs, which were all housed together in a single pen in the KSU Biosecurity Research Institute Biosafety Level 3Ag space. They were infected intranasally with a dose of  $7.66 \times 10^3$  TCID<sub>50</sub> of CSFV Paderborn. Clinical signs were monitored daily throughout the study and graded based on a scale modified from Mittelholzer et al. (2000) (see

Table 2.1). Rectal temperatures were taken daily after pigs first started exhibiting clinical signs (9 DPI). Veterinarians from the KSU Comparative Medicine Group were consulted to formulate treatment plans based on daily health observations. Intramuscular flunixin meglumine (Banamine 50 mg/ml; Merck Animal Health) was administered for pyrexia. Some pigs were given multiple courses of Banamine (3 consecutive days at a time), due to relapse in fever. Pigs with prolonged respiratory signs were given IM oxytetracycline (Liquamycin LA 200; Zoetis Inc) to limit development of secondary infections. If the clinical condition and pyrexia did not improve after Banamine therapy, they were humanely euthanized by intravenous Pentobarbital injection following the guidelines of the American Veterinary Medical Association. All efforts were made to minimize the stress and suffering of the pigs.

#### Sample collection

Oral fluid samples were collected daily as a pooled sample as described by Prickett et al., (2008). Briefly, braided cotton ropes were hung off the ground (bottom of rope was at shoulder level for pigs) in 3 separate locations in the pen; pigs were allowed to chew the ropes for 15-20 min. Fluid from all three ropes was collected and pooled. Individual serum samples were collected via jugular venipuncture from each pig at 0, 3, 6, 10, 14, 20, 28, and 31 DPI. Both OF and serum were stored at -80°C before being analyzed.

#### RNA Extraction

Extraction of RNA from serum and OF was performed using MagMax-96 Viral RNA extraction kit as per manufacturer's protocol (ThermoFisher Scientific). Briefly, 50 µl of sample was added to magnetic beads and incubated with lysis buffer. Bead bound nucleic acids were washed with two separate washing solutions (2 washes per solution; 4 total washes); nucleic acids were eluted with the provided buffer. Four serial 1:10 dilutions of virus stock were

prepared in nuclease-free water and extracted on the same plate as the sample RNA; these acted as the positive controls for both the extraction technique and the subsequent PCR analysis. Five microliters of extracted RNA were utilized for all PCR reactions.

#### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

We employed two separate reverse-transcriptase quantitative PCR protocols. The first one utilized the primer/probe, and thermocycler set-up described by Risatti et al. (2003). The reagent mix came from the AgPath-ID One-Step RT-PCR Reagents kit (Thermo Fisher). This protocol was used on both serum and OF to compare their efficiency in detecting CSFV in a population. The second protocol was a commercial kit offered by Tetracore; this is a lyophilized mixture containing primer/probe, and enzymes that is rehydrated with a supplied buffer. The Tetracore kit was only used with OF, to show this sample can be utilized with commercial kits optimized for serum analysis. A negative control (nuclease free water) was utilized on each PCR run. All PCR reactions were carried out on the BioRad CFX96 Touch Real-Time PCR Detection system for 45 amplification cycles. Serum samples and the OF sample used with the Tetracore kit were run in triplicate, OF samples analyzed with the Risatti protocol were run in duplicate.

To calculate  $\log_{10}$  TCID<sub>50</sub> equivalents/ml we created a standard curve by graphing a scatter plot of the cycle threshold (Ct) values of the four serial dilutions of virus stock as described by Weesendorp et al. (2009) (Figure 2.1). The fluorescence threshold was automatically determined by the thermocycler for each PCR. From the standard curve we could generate a linear regression equation:  $y = a + bx$ , where  $b$  is the slope,  $a$  is the y-intercept, and  $x$  and  $y$  are the independent and dependent variables, respectively. The generated equation gives us the slope, y-intercept; by setting the sample Ct as the dependent variable, we solve for the independent variable, which gives us  $n \log_{10}$  TCID<sub>50</sub> equiv/ml.

### Virus neutralization assay

Virus neutralization (VN) assays were performed on the last available serum sample collected for each animal, and on oral fluid samples corresponding with each bleed day (starting on 10 DPI). Once pigs with neutralizing sera were identified, previous bleed days were also analyzed to track development of neutralizing activity. Oral fluids were filter sterilized by passing through a 0.22 µm filter. Serum and filtered oral fluids samples were heated at 56°C for 30 min to inactivate protein complement, which can affect the neutralizing activity of antibodies. Samples were 2-fold serially diluted in sterile, supplemented MEM media (Gibco) on 96-well plates starting with an initial dilution of 1:8 and ending with a 1:1024 dilution. All samples were assayed in duplicate. CSFV Paderborn was added to each well of the diluted samples (20 TCID<sub>50</sub>/well) and incubated for 1 hour at 37°C, 5% CO<sub>2</sub>. The sample and virus mixtures were then transferred to 96-well plate of PK-15 cells at 60-70% confluence. After 1 hour the media on the cells was discarded and replaced with fresh, supplemented MEM. With each set of VN plates we included a set of back-titrations of the virus stock used to inoculate the plates.

### Immunofluorescence assay (IFA)

IFA must be performed to visualize CSFV in cell culture experiments (titration and VN), since the virus does not induce any changes in infected cells (i.e. cytopathic effect) noticeable with direct microscopy. After 3 days of incubation, the infected cell media was discarded, and adherent cells were rinsed three times with PBS. They were then fixed with 100% cold methanol and incubated at -20°C for 20 minutes. The methanol was then discarded, and the plates were air dried before being washed three times with PBS. We utilized both polyclonal pig sera (1:1000 dilution) and monoclonal antibody (mAb) WH303 (diluted 1:2000 in MEM) for primary labelling on IFAs. Polyclonal pig serum was supplied from a vaccine study in which pigs were

immunized with an Alphavax replicon particle expressing CSFV E2 antigen based on the Riems strain; it was only used for the initial virus titration experiment. The secondary antibody was AlexaFluor 488 IgG anti-pig or anti-mouse (Invitrogen) for the polyclonal serum or WH303, respectively. Both primary and secondary antibodies were incubated on fixed cells for 1 hour at 37°C; after each labelling step, the cells were rinsed with PBS. DAPI stain was added for 5 minutes at room temperature to provide nuclear staining. Stained plates were visualized on a EVOS fluorescent microscope (Thermo Fisher).

### Protein expression

The DNA sequence of the E2 ectodomain (nucleotides 1-1008) was commercially synthesized by Genscript, and cloned into the Pet28a vector using restriction enzymes EcoRI and Sall (New England Biolabs). The vector was then transformed into BL21 DE3 cells (New England Biolabs). Protein expression was performed over 4 hours in 500 ml of 2XYT medium (Sigma Aldrich), and induced using 0.4 mM of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). The bacteria were pelleted by ultracentrifugation, and the pellet was then lysed using 1x LEW Buffer (USB Corporation), protease inhibitor cocktail (Thermo Fisher) and lysozyme (Sigma Aldrich). After 30 minutes on ice in this solution, mechanical lysis was also performed with a 3000 Ultrasonic Homogenizer (Biologics). This was followed by a second centrifugation step, and the resulting pellet was resuspended in 1x lysis equilibrium wash buffer (LEW) + 8M Urea. This solution was centrifuged a third time, and the supernatant was used to purify E2 protein under denaturing conditions using the MIDI-Kit as per manufacturer's instructions (Thermo Fisher). A more thorough explanation of cloning and bacterial expression is included in the "Methods" section for Chapter 3.

### Bovine Serum Albumin (BSA) protein assay

To determine the concentration of purified E2 protein, five separate dilutions of BSA (ranging from 500 µg/ml to 100 µg/ml) were prepared in BioRad Protein Assay Dye Reagent mix. The target protein was also diluted 1:2; samples and standards were read on the Epoch Biotek plate reader. This gives the 405-650 nm optical density (OD) of each sample; the OD of the standards were used to generate a standard curve. The concentration of the target protein was then calculated from the standard curve using the linear regression equation, as described above.

### Enzyme Linked Immunosorbent Assay (ELISA)

An indirect ELISA assay was constructed using the ectodomain of E2 as the bait antigen. Purified protein was diluted to 4 mg/ml in Carbonate Coating Buffer and used to coat 96-well flat bottom plate (Costar). After coating overnight at 4°C, the plate was blocked with Goat serum (diluted to 10% in PBS) for 1 hour at 37°C. The plate was then washed 3 times with PBS-Tween 20. Serum or oral fluids were diluted 1:10 and 1:2, respectively, and added for 1 hour at 37°C. After a second wash the secondary antibody (peroxidase-conjugated goat anti-swine; Invitrogen) was added for 30 min at 37°C. The plate was washed again, and a colorimetric reaction was induced using ABTS Microwell Peroxidase Substrate (KPL Laboratories). This reaction was stopped with 1% SDS solution. ELISA plates were visualized on Epoch Biotek plate reader, which provided optical density (OD) values. These values were used to calculate sample to positive (S/P) ratios; because no reliable positive control was available, Pig 43 serum (from 20 DPI of this study) was used. The S/P ratio is equal to

$$\frac{\text{average sample OD} - \text{average of negative control OD}}{\text{average of positive control OD} - \text{average of negative control OD}}$$

## Results

### Clinical signs and Gross Necropsy findings

The clinical condition began to deteriorate on 9 DPI in pigs 47 and 48; they presented with pyrexia (temperature >105°F), anorexia and rough coats. Pigs 46, 47, and 48 were euthanized on 15 DPI due to high clinical scores and failure to respond to therapy and deteriorating condition. These pigs were categorized as having subacute disease due to the rapid progression in their clinical signs (Figure 2.2a-c). After 14 DPI the other pigs also developed pyrexia, and clinical signs, such as, pyrexia, diarrhea, and anorexia being the most common. Pigs 39, 40, 41, and 44 initially responded to Banamine treatment, but their fever returned several days later and eventually became refractory to treatment. This resulted in a gradual reduction in the population from 24 to 31 DPI (Figure 2.5). We identified these pigs as having chronic disease due to late onset of disease and much more gradual deterioration in clinical signs (Figure 2.3a-d). Pig 39 (Figure 2.3a) was the final pig euthanized for welfare reasons on 31 DPI (as a result the study was terminated, since there were only a few pigs remaining). The final three pigs (42, 43, & 45) only developed mild fevers and clinical signs, which peaked between 15-20 DPI. All were in good health by the end of the study so they were labeled as Recovered/persistent (Figure 2.4a-c). Based solely on clinical sign grading, the pigs could be separated into three separate groups; the differences between groups can be seen in the PCR and antibody response results (Table 2.2).

At necropsy, all pigs (except 42 and 43) had diffuse hemorrhagic congestion in most lymph nodes, and multi-focal to diffuse ecchymoses and petechiae in the lungs. The subacute pigs presented with the most severe gross pathology relative to the other two groups (Table 2.2). Pig 39 was the only pig to develop any sort of skin hemorrhages, i.e. ecchymoses on the edges of both ears. Pig 47 had diffuse pleuritis and pericarditis, along with fibrinous adhesions. Pigs 47

and 40 had consolidation within the ventral portions of their lungs. Pigs 40 and 44 had diffuse petechiae on both kidneys. Pig 45 displayed ecchymotic streaking on the stomach, and had multifocal splenic infarcts.

#### Viremia via PCR on serum and OF

Virus was first detected in the serum of one pig on 6 DPI (Table 2.3). The Subacute group was the first to have detectable serum viremia (6 to 10 DPI). Pig 45 also had detectable viremia on 10 DPI, but the virus titers remained relatively constant, while in the Subacute group the Log<sub>10</sub> TCID<sub>50</sub> equiv/ml continued to rise until 14 DPI. Virus was detected in the rest of the pigs starting on 20 DPI. While the Log<sub>10</sub> TCID<sub>50</sub> equiv/ml increased later in the Chronic group versus the Subacute group, pigs in both groups had >6 Log<sub>10</sub> TCID<sub>50</sub> equiv/ml by the time they were euthanized. Virus levels remained low in the Recovered/persistent group (<5.5 Log<sub>10</sub> TCID<sub>50</sub> equiv/ml), and these pigs were able to clear the virus from serum by the end of the study. PCR could also be used to separate the pigs into three separate disease groups based on when virus was first detected and whether the pig had cleared it by the end of the study (Table 2.2).

Both the commercial and the Risatti et al. (AgPath kit) protocols yielded very similar results, first detecting virus in OF on 8 DPI (Figure 2.6). The fluctuation in Log<sub>10</sub> TCID<sub>50</sub> equiv/ml in the pooled OF sample mirrored the situation seen in serum. Levels first increased to an apex from 8 to 14 DPI, then decreased by 20 DPI as the sickest pigs were euthanized. The highest virus levels in OF were seen on 31 DPI. These results show that qRT-PCR was a sensitive method of detecting virus in OF, and did so one day before pigs became clinical.

#### Antibody response – VN and ELISA

Neutralizing activity was not detected in OF with VN on any day of the study. As shown in Figure 2.7, pigs 42, 43, and 45 developed neutralizing antibody levels that ranged from 1:256



to over 1:512. Neutralizing antibody titers were first detected at 10 DPI and increased before plateauing at 28 DPI. Pig 39 developed lower levels of neutralizing antibodies (1:64) and no other pigs had detectable amounts of neutralizing antibodies (data not shown). The results were similar on ELISA, with the 3 surviving pigs producing the highest titers of E2 specific antibodies as determined by S/P ratio (Figure 2.8). Pig 48 produced the greatest antibody titers out of the pigs that developed fatal disease; the other pigs produced low levels of antibodies. The levels in oral fluids barely rose above that of the negative control by 31 DPI. Antibody response could not be assessed in OF using ELISA or VN assay; however, when performed with serum these assays could separate the Recovered/persistent group from the other pigs (Table 2.2).

## **Discussion**

The clinical observations showed the development of three separate disease states upon infection with CSFV Paderborn. As outlined in Table 2.2, differentiating factors between the three groups were identified through clinical observation, qRT-PCR, and antibody response. The clinical scoring showed that pigs succumbing to subacute disease deteriorated quickly, despite therapy, while the group that had a chronic disease presentation showed initial improvement in temperature and clinical signs (Figure 2.2 and 2.3). The latter group all exhibited several cycles of fever and recovery, before finally deteriorating to the point where they had to be euthanized. The Recovered/persistent group saw a single spike in temperatures and clinical signs between 15-20 DPI, but then recovered without redeveloping any further disease (Figure 2.4). It is also important to note that in the literature chronic infection is described as beginning after 30 days and as far as 2 months post initial infection (Van Oirschot et al., 1983; Moennig et al., 2003; Blome et al., 2017). Therefore, it is completely possible that the pigs we observed to be healthy at the end of the study may have relapsed and developed persistent chronic infection if the study

extended for several months. We use the descriptors of subacute, chronic and recovered/persistent to differentiate the three groups relative to one another. Necropsy findings were consistent with other reports from moderate/chronic CSFV infection studies (Cheville and Mengeling, 1969; Moennig et al., 2003; Blome et al., 2017).

The differences between the groups was further accentuated on qRT-PCR; Table 2.3 shows how after developing high titers ( $6 \log_{10}$  TCID<sub>50</sub> equiv/ml) pigs had to be euthanized. Conversely, the pigs that recovered never had titers above  $5.33 \log_{10}$  TCID<sub>50</sub> equiv/ml. The latter group had undetectable levels of virus in serum on the final day. Comparing the results from clinical scoring with the virus levels in Table 2.3 shows a positive correlation between increase in titers and deterioration in clinical condition. These results were mirrored on the qRT-PCR results from OF samples. Virus levels increased as the Subacute group's clinical condition deteriorated, and reached even higher levels once the Chronic group began to develop terminal disease (Figure 2.6).

Virus was first detected in OF on 8 DPI, which is when elevated levels of virus are found in all body secretions (Weesendorp et al, 2009). Weesendorp et al. (2009) also showed virus levels peaking in OF samples after 30 days. Similarly, the highest  $\log_{10}$  TCID<sub>50</sub> equiv/ml was recorded on 31 DPI, when only 1 pig had detectable serum viremia. This is due to a persistence and continued shedding from tonsils (Cheville and Mengeling, 1969; Tarradas et al., 2014), despite clearance from serum. Dietze et al. (2017) reported positive results on 7 DPI in pooled OF, but they used a higher infectious dose and a more virulent strain (Alfort/Tübingen) (Aynaud et al., 1972). Moreover, they first detected virus in serum on 2 DPI, indicating a greater discrepancy between serum and OF detection on qRT-PCR. We only saw a two day gap between serum and OF detection; this difference is likely due to different excretion patterns for strains of

different virulence (Weesendorp et al., 2009). Altogether, these results are very promising since they show that OF can detect CSFV very early in infection and can track the virus in a population using fewer samples and resources. Additionally, this method can track persistence in OF after serum clearance, which has not been shown for pooled OF sampling for CSFV. While serum sampling detected virus more quickly in this study, it is important to remember that in a large pig barn with hundreds or thousands of pigs, serum sampling of every animal is impossible, especially over extended periods of time. Studies with PRRSV have shown that the sensitivity of OF sampling reaches 90-100% sensitivity once disease prevalence is at 30% or above (Olsen et al., 2013; De Regge and Cay, 2016). With influenza, the prevalence needs only 18% for a detection probability of 99% (Romagosa et al., 2012). With either virus, pooled OF samples provided an improvement over single-animal sampling. Similar studies must be performed with CSFV to determine the probability of detecting the virus given varying prevalence under field conditions.

One disadvantage that has been documented both in this study and by Petrini et al. (2017), is a lack of detectable CSFV-specific antibodies in OF. Antibodies against PRRSV, influenza and PCV2 can be detected in OF within two weeks post infection (Prickett et al., 2008; Kittawornrat et al., 2012; Panyasing et al., 2013). Neutralizing antibodies have been detected in the oral fluids of CSFV-infected swine only 30 days post immunization with the CSFV Thiverval vaccine strain (Corthier and Aynaud, 1977). In the current study, only three pigs had elevated levels of E2-specific antibodies at one time, as determined by ELISA (Figure 2.8). It is unclear why no antibodies were detected in OF in the current study. Possibilities include dilution of antibodies in OF, or presence of substances in OF interfering with antibody binding. Neutralizing antibodies in serum appeared only in 4 pigs, with the same three Recovered/Persistent pigs

displaying high titers (Figure 2.7). Neutralizing antibodies have been shown to provide good protection against CSFV infection (Terpstra and Wensvoort, 1988; van Gennip et al., 2000). The development of these antibodies on 10 DPI and their steady increase until 28 DPI is in accordance with other reports in the literature (Petrov et al., 2014; Tarradas et al., 2014). Depner et al. (1996) also reported pigs that recovered from moderate CSFV infection with high neutralizing antibody titers and minimal clinical signs. However, their study was carried out to 90 days, during which time they had a pig develop neutralizing antibodies and then subsequently lose them; this pig died from CSFV-related disease at 71 DPI. It is unclear whether all the pigs we deemed recovered would have maintained their neutralizing antibodies and truly clear the infection, or whether the disease would relapse months down the road.

We successfully implemented an infectious model, which displayed all three disease presentations associated with moderate CSFV disease. We also showed that OF rope sampling can be an effective method of detecting and tracking disease progression in 10 pigs infected under our model. Future studies must focus on implementing these methods in the field on large pig farms to determine their sensitivity and specificity in detecting CSFV. This study is an important first step in improving mass population surveillance for CSFV.

### **Acknowledgements**

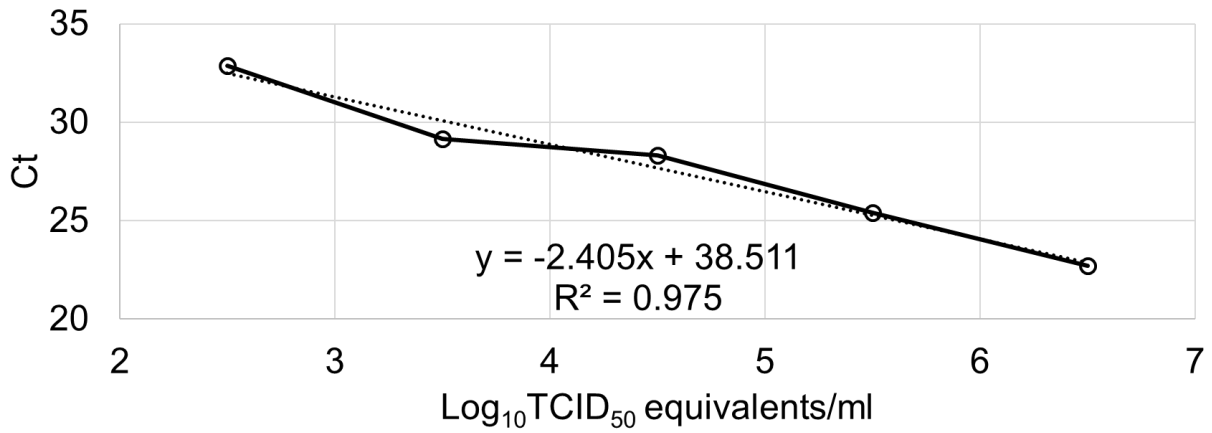
*This research was supported by the State of Kansas National Bio and Agro-Defense Facility fund.*

<b><u>Table 2.1 Clinical sign grading chart</u></b>					
<b><u>Attitude</u></b>	<u>0</u> - BAR	<u>1</u> - Quiet, but alert	<u>2</u> - Depressed, gets up slowly	<u>3</u> - Obtunded, won't get up	
<b><u>Respiration</u></b>	<u>0</u> - 10-15 breaths/min	<u>1</u> - >20 breaths/min. Sneezing occasionally	<u>2</u> - >20/min, obvious effort. Coughing regularly.	<u>3</u> - 30/min, open mouth	
<b><u>Body Condition</u></b>	<u>0</u> - filled out	<u>0.5</u> - rough hair coat	<u>1</u> - empty stomach,	<u>2</u> - muscle wasting	<u>3</u> - ribs and vertebrae are prominent
<b><u>Feces</u></b>	<u>0</u> - normal	<u>1</u> - reduced, dry	<u>2</u> - small amount, OR diarrhea	<u>3</u> - no feces, OR watery/bloody diarrhea	
<b><u>Eyes</u></b>	<u>0</u> - normal	<u>1</u> - reddened, clear discharge	<u>2</u> - highly inflamed, turbid discharge	<u>3</u> - highly inflamed, purulent discharge, thick blood vessels	
<b><u>Skin</u></b>	<u>0</u> - normal	<u>1</u> - red areas; clear discharge	<u>2</u> - purple/cold areas, few petechia; turbid discharge	<u>3</u> - black/red areas, large hemorrhages; purulent secretions	<u>W</u> - wound from activity in pen, not CSFV
<b><u>Nervous signs</u></b>	<u>0</u> - normal	<u>1</u> - hesitant, slow corrections.	<u>2</u> - ataxia/lameness, but walking. Tremors	<u>3</u> - unable to stand, disoriented	
<b><u>Lameness</u></b>	<u>0</u> - normal	<u>1</u> - lame, weight bearing	<u>2</u> - lame, toe-touching	<u>3</u> - non-weight bearing lame	

**Table 2.1 Clinical sign grading chart**

Each pig was assessed daily and assigned a score from 0 to 3 for each category, with 0 being normal and 3 being the most abnormal. The overall clinical score and temperature were considered when determining the need for treatment or euthanasia.

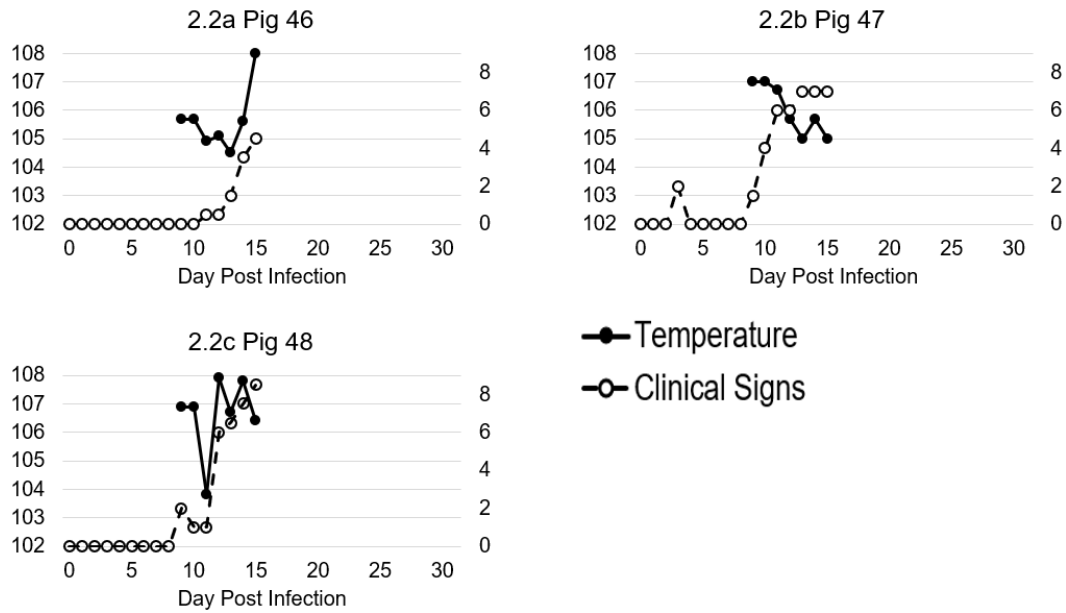
Figure 2.1 Standard Curve Plot



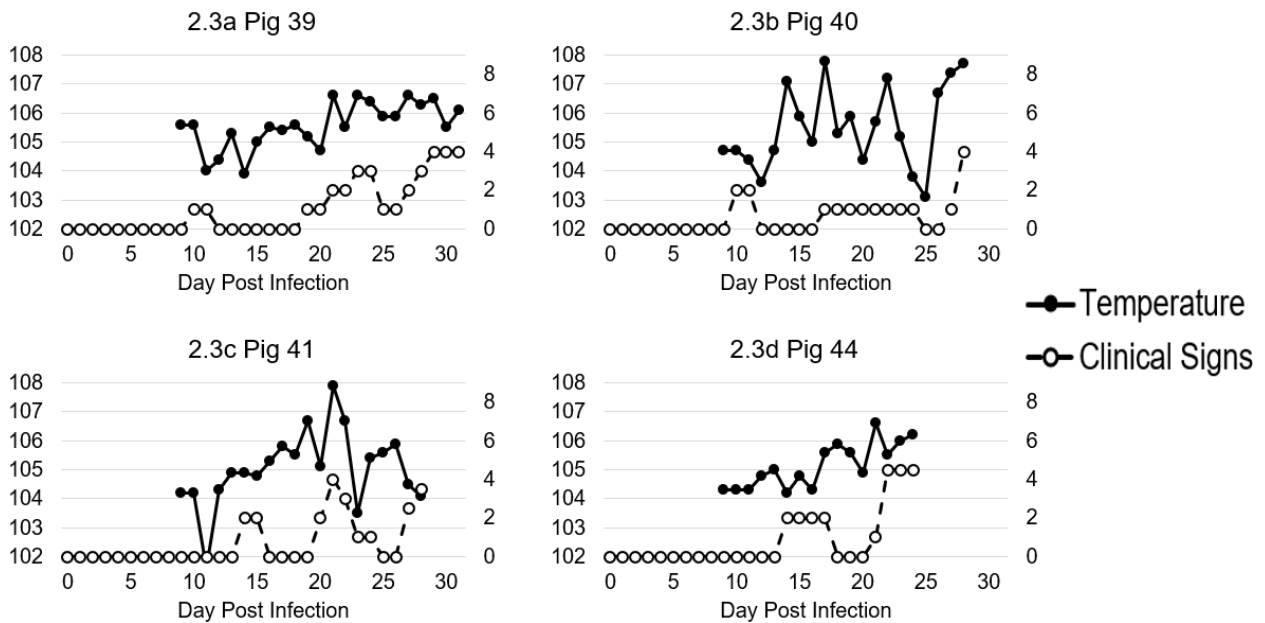
**Figure 2.1 Standard Curve for qRT-PCR**

Example of standard curve generated from serial dilutions of virus stock used to calculate Log<sub>10</sub> TCID<sub>50</sub> equivalents/ml.

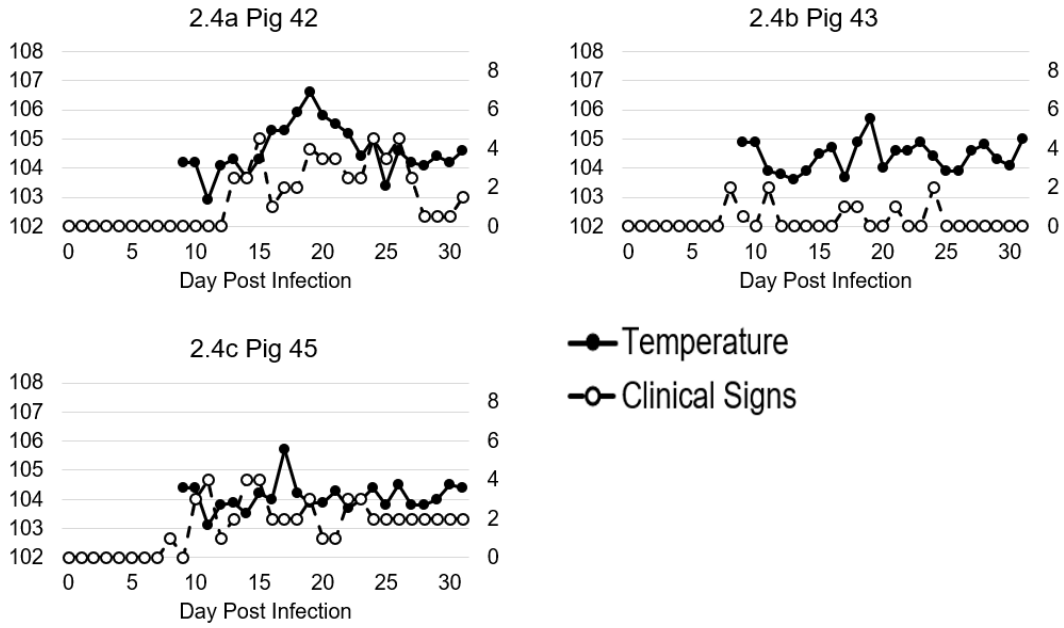
**Figure 2.2 Clinical scores and Temperatures – Subacute Group**



**Figure 2.3 Clinical Scores and Temperatures – Chronic Group**

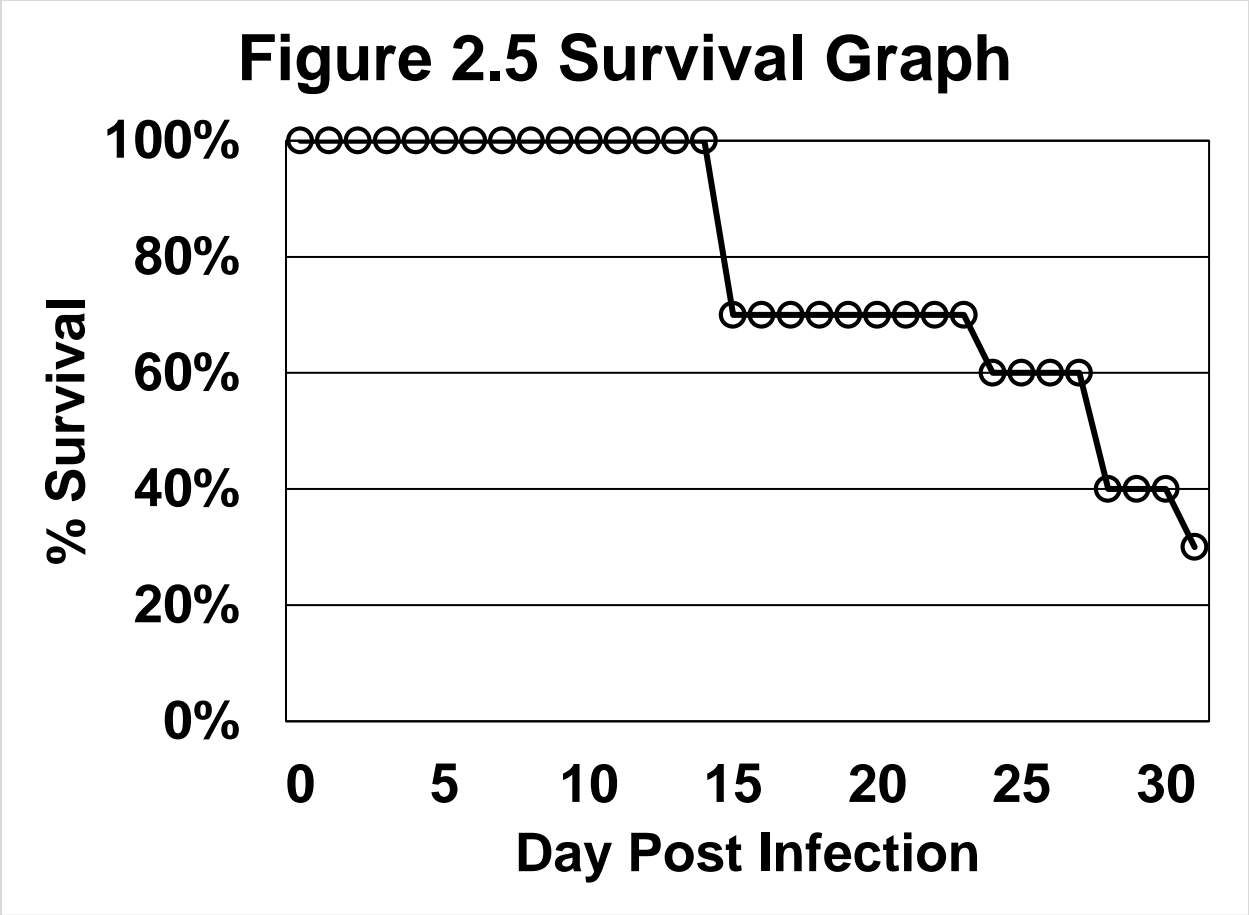


**Figure 2.4 Clinical Scores and Temperatures – Recovered/Persistent Group**





<b>Table 2.2</b> Breakdown of CSFV disease states			
	<b>Subacute</b>	<b>Chronic</b>	<b>Recovered/Persistent</b>
<b>Pigs</b>	<b>46; 47; 48</b>	<b>39; 40; 41; 44</b>	<b>42; 43; 45</b>
<b>Disease course</b>	Deterioration starting at 9 DPI, euthanized at 15 DPI. Severe gross pathological findings.	Multiple spikes in clinical signs and temperatures; euthanized between 24-31 DPI. Moderate-severe gross pathological findings	Single spike in fever/clinical signs around 15 DPI. No clinical signs at 31 DPI. Mild to moderate gross pathological findings.
<b>Response to therapy</b>	None	Initial response, then became refractory	Full response, no relapse in disease
<b>PCR results</b>	1 <sup>st</sup> detected 6-10 DPI >6 Log <sub>10</sub> TCID <sub>50</sub> equiv/ml	1 <sup>st</sup> detected 20 DPI >6 Log <sub>10</sub> TCID <sub>50</sub> equiv/ml	1 <sup>st</sup> detected 20 DPI Cleared from serum by 31 DPI
<b>Antibody response</b>	Minimal E2 specific antibodies, no neutralizing activity	Minimal E2 specific antibodies, limited neutralizing activity	All developed high titers of neutralizing antibodies



**Figure 2.5 Survival Graph**

Survivability graph shows the three clinical presentations: subacute (euthanized at 15 DPI), quick deterioration without response to therapy; chronic (euthanized between 24-31 DPI), initial response to therapy but eventual deterioration; recovered/persistent, these pigs had no apparent clinical signs at 31 DPI.

<b>Table 2.3 Serum qRT-PCR</b>							
<b>Pig</b>	<b>Day 3</b>	<b>Day 6</b>	<b>Day 10</b>	<b>Day 14</b>	<b>Day 20</b>	<b>Day 28</b>	<b>Day 31</b>
<b>46</b>	UND	UND	4.56	6.67			
			0.17	0.09			
<b>47</b>	UND	UND	5.65	7.80			
			0.14	0.10			
<b>48</b>	UND	3.98	7.22	8.55			
		0.18	0.04	0.06			
<b>39</b>	UND	UND	UND	UND	6.45	8.36	7.59
					0.05	0.03	0.06
<b>40</b>	UND	UND	UND	UND	6.31	7.81	
					0.05	0.04	
<b>41</b>	UND	UND	UND	UND	7.03	9.26	
					0.01	0.04	
<b>44</b>	UND	UND	UND	UND	6.32		
					0.05		
<b>42</b>	UND	UND	UND	UND	3.33	5.33	UND
					0.20	0.04	
<b>43</b>	UND	UND	UND	UND	2.73	4.27	UND
					0.74	0.22	
<b>45</b>	UND	UND	5.05	4.65	1.88	3.80	UND
			0.13	0.10	0.40	0.07	

**Table 2.3** Results given as average  $\text{Log}_{10}$  TCID<sub>50</sub> equiv/ml of three replicate PCRs, with standard deviation below. UND = undetermined, did not cross the fluorescence threshold after 45 amplification cycles. Pigs are ordered by clinical group from top to bottom: subacute (red), chronic (yellow), and recovered/persistent (green).

Figure 2.6 Oral Fluid qRT-PCR

### Figure 2.6 Oral Fluid qRT-PCR

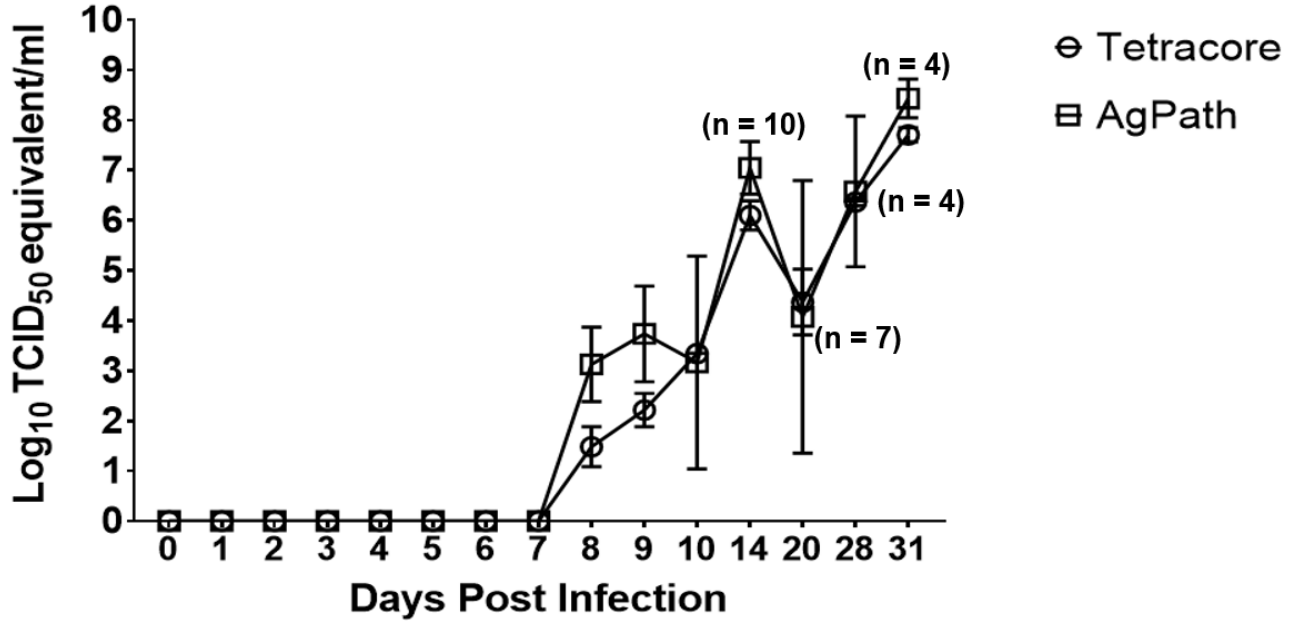
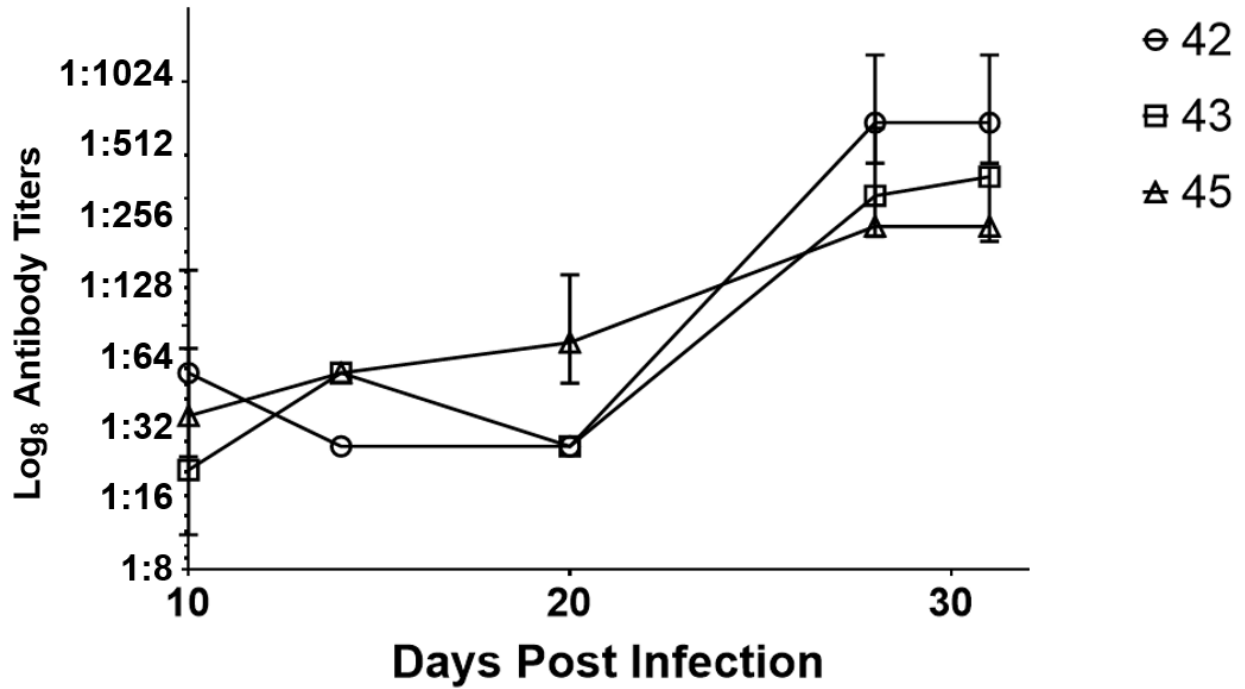


Figure 2.6 Oral Fluid qRT-PCR Results given as averages (3 replicates for Tetracore; 2 replicates for AgPath) with standard deviation. Values plotted at zero did not cross the fluorescence threshold after 45 amplification cycles. The “n” value indicates number of pigs comprising the total population at the respective day post infection.

**Figure 2.7 Virus Neutralization assays**



**Figure 2.7 Virus Neutralization assay**

Serum neutralization antibody levels shown as Log<sub>8</sub> titers over 31 days in the three of the Recovered/persistent group.

## Figure 2.8 ELISA by Clinical Group

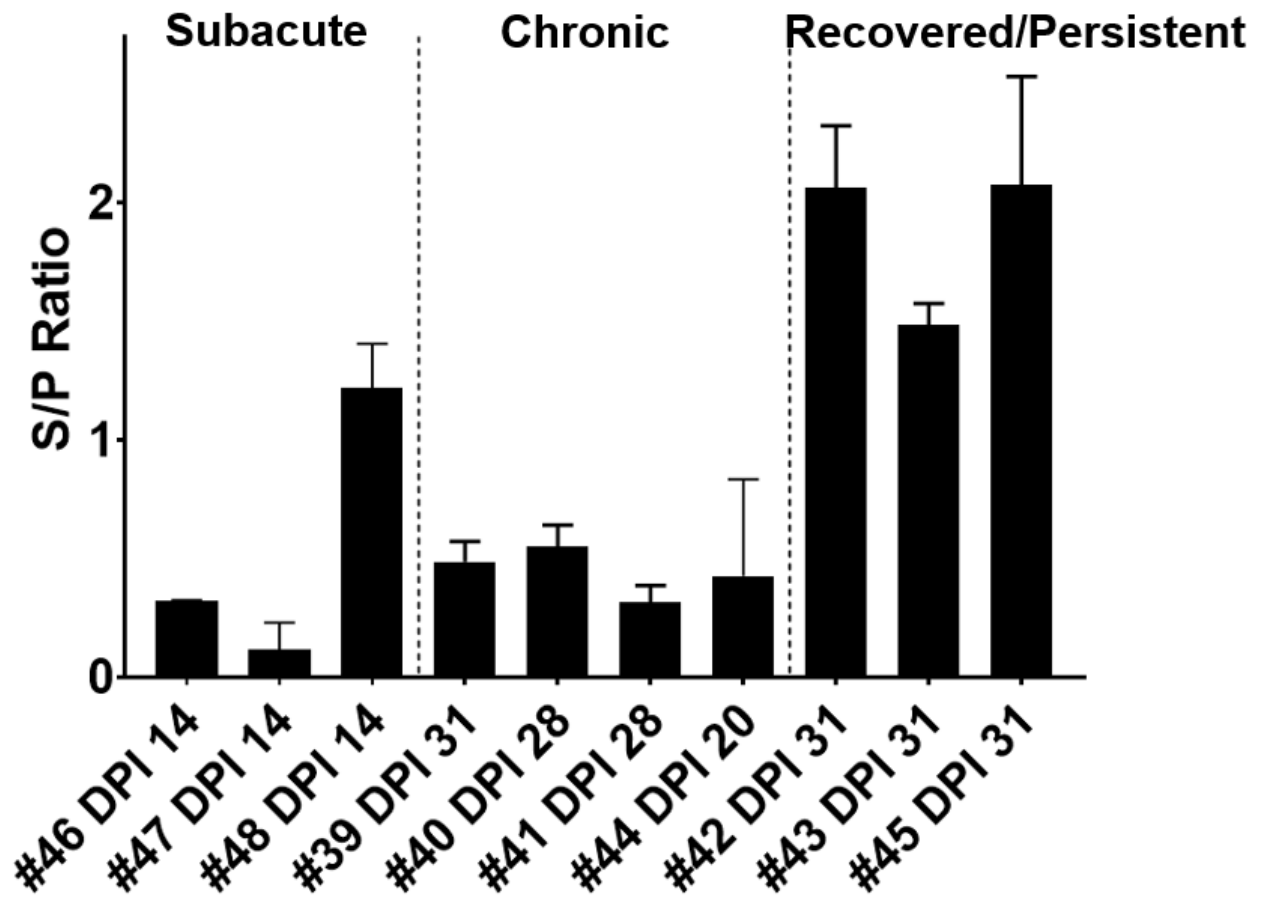


Figure 2.8 ELISA of Serum samples

Individual ELISAs of last available serum sample for individual pigs.

# **Chapter 3 - Developing monoclonal antibodies for Classical Swine Fever virus antigens, E2 and Erns**

## **Introduction**

Classical swine fever virus (CSFV) belongs to the family Flaviviridae, genus Pestivirus. Members of this genus utilize four structural proteins (E1, E2, Erns and Core protein) and seven nonstructural proteins (Npro, NS2, NS3, NS4A, NS4B, NS5A, NS5B) to establish infection (Lindenbach et al., 2007; Schweizer and Peterhans, 2014). Of these proteins, E2 and Erns are the most antigenic and immunogenic, making them ideal antigens for generating monoclonal antibodies (mAbs) used in diagnostic assays (Gavrilov et al., 2011). Antibody production begins when antigens from invading pathogens are processed by dendritic cells, which then stimulate T cells; together, these two cells select the B cells that have receptors that bind the chosen antigen. The few B cells that do this with sufficient affinity are then stimulated to undergo somatic hypermutation (Banchereau and Steinman, 1998; Mantegazza et al., 2013; Nierkens et al., 2013). This mechanism allows B cells to acquire increased affinity for the stimulating antigen, and in return, they produce high affinity antibodies that are reactive to a specific epitope on the given antigen (Chan and Brink, 2012). This same principle is utilized to develop monoclonal antibodies (mAbs) in mice to target very specific targets for therapies or diagnostic assays (García Merino, 2010). They are used in all CSFV diagnostic techniques, except for polymerase chain reactions (PCR) (Hoffmann et al., 2005; Risatti et al., 2005; Le Dimna et al., 2008; Dias et al., 2014).

Techniques like virus isolation (VI) and virus neutralization assays (VN) are the most sensitive and specific and are the confirmatory test of choice. These assays typically employ

fluorescently conjugated mAbs to visualize virus-infected cells. Their disadvantage is that they require 3-4 days to allow virus growth on permissive cells (Grummer et al., 2006; “Access online,” 2014). Serological assays, like enzyme-linked immunosorbent assay (ELISA), are the most widely used screening tests for CSFV. Competitive ELISAs are preferred to direct/indirect ELISAs since they utilize competition between a monoclonal antibody (mAb) and antibodies in test sera binding to a supplied antigen (e.g. E2 or Erns) (Greiser-Wilke et al., 2007; “OIE Terrestrial Manual” 2014). This competitive binding principle increases specificity of the assay and reduces the cross-reactivity seen with simpler ELISAs. The complex trapping and blocking (CTB) ELISA takes this a step further by having an initial trapping mAb that is immobilized to the plate and captures E2 antigen; then a second mAb targeting a separate E2 epitope is added at the same time as test sera (Wensvoort et al., 1988). Thus, antibodies from test sera must compete with two mAbs, which limits non-specific binding. This ELISA was commercialized and used extensively during the 1997-98 CSFV outbreak in the Netherlands; however, due to cross-reactivity to other Pestiviruses (i.e. bovine viral diarrhoea virus), the results had to be confirmed by VN assay (de Smit et al., 2000). Pigs often have antibodies against BVDV in the field, so any serological test used for surveillance, must be highly specific for CSFV (Ridpath, 2010; Tao et al., 2013).

In CSFV-endemic regions, like Asia and Latin America, accurate surveillance is important to direct vaccination programs trying to limit disease impact (Terán et al., 2004; Pereda et al., 2005; Luo et al., 2014; Blome et al., 2017). Meanwhile, in disease free regions (e.g. US and Europe) surveillance efforts are meant to ensure that the virus has not been introduced (Sell and Watson, 2013; “USDA APHIS | 2013 Program Data Report A - Resource Category Listing of WS Operations Line Item (Including HPAI) Funding and Cooperative Funding,” 2013,



“Funding of Animal Health Measures - Food Safety - European Commission,” n.d.).

Surveillance efforts are heavily dependent on veterinarians initially identifying the disease in the field and collecting samples for confirmatory testing (de Smit et al., 2000; Fritzscheier et al., 2000; Elbers et al., 2002; Engel et al., 2005). Due to the ability of CSFV to cross geographical borders and in producing heavy economic losses, a rapid and specific serological test is essential for control, prevention and in eradication programs. The approach of this study was to develop, characterize and evaluate mAbs against immunogenic CSFV glycoproteins, E2 and Erns. Our goal is to incorporate these mAbs into a blocking ELISA and improve upon the sensitivity and specificity of current serological assays.

## **Methods**

### Cloning

To produce specific monoclonal antibodies to the CSFV structural proteins, E2 and Erns DNA sequences were produced commercially (Genscript). The Paderborn strain was used as the reference genome; the E2 gene spanned nucleotides 1-1002, and the Erns gene spanned nucleotides 1-504. These regions are flanked by restriction sites for EcoRI and SalI, and omit the C-terminal transmembrane domains and amphipathic helix of E2 and Erns, respectively. These gene fragments were cloned into pET vector 28a and transformed into Top 10F' cells (Invitrogen). In brief, purified DNA is added to competent cells on ice and incubated for 30 minutes. The cells are then heat shocked for 30 seconds in 42°C and then grown in (SOC) media for 30-45 minutes. They are then added to Luria-Burlani (LB) agar plus Kanamycin plates and cultured overnight. Colonies are selected from the plate and grown overnight, and DNA is

purified from these cultures using the PureYield Plasmid Miniprep system as per manufacturer's instructions (Promega).

Both plasmids were verified via restriction digestion with *EcoRI* and *SalI* enzymes, and analyzed on a 1% Agarose gel; the UVP GelDoc-It Imaging system was used to visualize and photograph the gels. The resulting bands were purified (Promega SV Gel & PCR Clean-up kit) and sent for sequencing (ACGT Inc.) to confirm correct sequence and in frame orientation within the plasmid. Sequences were aligned and compared using CLC Main Workbench; this program was also used to align our sequences to reference genomes from Genbank. The proteins were then transformed into competent BL21 DE3 cells following the same protocol as for the Top 10 F' cells (New England Biolabs).

#### Protein Expression and Purification

Protein expression was performed over 4 hours in 500 ml of 2XYT medium (Sigma Aldrich) at 37°C, and induced using 0.4mM of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). The bacteria were pelleted, and the pellet was then lysed using B-PER, lysozyme and phenylmethane sulfonyl fluoride (PMSF). After 30 minutes on ice in this solution, mechanical lysis was also performed with a 3000 Ultrasonic Homogenizer (Biologics). Then the proteins were bound to Ni-NTA beads and purified using Urea buffers of increasing acidity (from pH 6.3, 5.9, and 4.5). The purified protein was dialyzed (using Spectra/Por dialysis tubing) and concentrated (using polyethylene glycol flakes; Milipore Sigma), before being electroeluted for further purity and specificity. The electroeluted protein was again dialyzed and concentrated. Dialysis aids refolding of the protein and removing urea residues, to ensure a secondary structure as similar as possible to the native protein. Concentrating the protein aids in the subsequent immunization steps. SDS-PAGE gels (12.5%) were used to verify the expression and

purification; Western blots (see protocol below) were used to verify the correct protein was isolated. Gel and western blot pictures were taken on the UVP GelDoc-It Imaging system.

#### Recombinant polypeptide E2 fragments

To determine what regions were targeted by E2 antibodies, we synthesized DNA gene fragments (Genscript) spanning nucleotides 1-300, 300-600, and 600-1002 of the N-terminal portion of E2. These plasmids were cloned into a pHUE vector using restriction enzymes as described above. Expression was carried out in Luria-Burlani media, and induced with 0.4mM of IPTG. Purification was performed under denaturing conditions using the PrepEase Hisitidine-tagged protein purification kit (Affymetrix) following the manufacturer's instructions. These proteins were then used in Western blots (see below).

#### Bovine Serum Albumin (BSA) protein assay

To determine the concentration of purified E2 and Erns proteins, five separate dilutions of BSA (ranging from 500 µg/ml to 100 µg/ml) were prepared in BioRad Protein Assay Dye Reagent mix. The target protein was diluted 1:2; samples and standards were read on the Epoch Biotek plate reader. The optical density (OD) of the standards were used to generate a standard curve. The concentration of the target protein could then be calculated from the standard curve using the linear regression equation:  $y = a + bx$ , where b is the slope, a is the y-intercept, and x and y are the independent and dependent variables, respectively.

#### Monoclonal antibody production

Experiments involving animals and virus were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the United States Department of Agriculture Animal Welfare Act and

Animal Welfare Regulations, and were approved by the Kansas State University (KSU) Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

The workflow for generating mAbs is illustrated in Figure 3.1. Purified proteins were diluted in PBS and mixed with Freund's incomplete adjuvant (Sigma Aldrich), so that each mouse received 100 µg of purified protein. We utilized 3 BALB/c (Charles River) mice per protein, each being immunized every 2 weeks, for a total of 3 cycles. Mice were monitored several times per day to ensure they did not react too severely to immunization. Two weeks after the third immunization the mice were sacrificed, and their spleens were harvested. The spleen was mechanically macerated to isolate the antibody-producing from the splenic parenchymal cells. The splenocytes were washed with MEM media and then fused to immortalized myeloma cells using polyethylene glycol solution in a 37°C-water bath. The fused cells were distributed on 24-well plates and grown for several days until colonies of fused hybridoma cells began to appear. These cells were grown using hypoxanthine-aminopterin-thymidine (HAT; Thermo Fisher) media, which essentially blocks de novo DNA synthesis in unfused cells. The splenocytes have the appropriate enzymes to survive off the HAT media, but only live for a few days, while the myeloma cells are immortal, but do not have the proper enzymes. Thus, only fused cells may survive long enough to be selected. Each well was screened via IFA on a 96-well plate on CSFV-infected cells. The cells collected from the positive wells of the 24-well plate are serially diluted on 96-well plates to try to isolate a single clone that produces a monospecific antibody.

#### Indirect Immunofluorescence assay (IFA)

IFA must be performed as a screening method for the reactivity of hybridoma clones to the detect CSFV antigen. To do this we infected half of a 96-well plate seeded PK15 cells with

20 TCID<sub>50</sub>/well of CSFV Paderborn strain. The uninfected half acts as a negative control to test for non-specific binding in hybridoma supernatant. The PK15 cells were maintained in MEM culture media (Life Technologies) supplemented with 7% fetal bovine serum, L-glutamine, 0.01% Penicillin/Streptomycin, and 0.008% Amphotericin-B (Gibco) in a 37°C 5% CO<sub>2</sub> incubator. Three days after viral infection, the infected cell media was discarded, and adherent cells were rinsed three times with PBS. They were then fixed with 100% cold methanol and incubated at -20°C for 20 minutes. The methanol was then discarded, and the plates were air dried before being washed three times with PBS. Undiluted hybridoma supernatant was incubated on fixed cells for 1 hour at 37°C. After washing with PBS, a second incubation for 1 hour at 37°C was performed with the secondary antibody (1:800), AlexaFluor 488 IgG anti-mouse (Invitrogen). The well characterized mAb WH303 was used as a positive control on all infected plates, at a dilution of 1:2000 (Lin et al., 2000). Stained plates were visualized on a EVOS fluorescent microscope (Thermo Fisher).

### Western blot

Western blotting was used to analyze the reactivity of mAbs to E2 fragments in order to map their position. The fragments migrated on a 12.5% SDS-PAGE gel, and then electroblotted on a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 10% Goat serum in PBS overnight at 4°C. It was then stained with monoclonal (diluted 1:50 in PBS + Goat serum) or anti-His (diluted 1:1000 in PBS + Goat Serum) antibodies at 37°C for 1 hour. After washing 3 times with PBS Tween-20, a peroxidase labeled, goat anti-mouse antibody (Sera care) was diluted 1:4000 in PBS + Goat Serum and incubated on the membrane at 37°C for 1 hour. The membrane was washed again and stained using CN/DAB substrate (Thermo Fisher) as per manufacturer's instructions.

## Results

### Cloning and Protein Expression

Agarose gel electrophoresis demonstrated correct band size for E2 and Erns inserted into pET28a vector (Figure 3.2); products were run in duplicate to assure sufficient DNA for subsequent transformation reactions. Sequencing results from ACGT were compared to the CSFV Paderborn reference genome and confirmed to be identical and in the proper orientation in the pET28a vector. These gene fragments were then expressed in BL21 DE3 cells and purified under denaturing conditions (Figure 3.3). Denatured proteins were refolded using dialysis and concentrated using PEG flakes. They were then purified a second time using electroelution; this was followed by a second refolding and concentration step. Finally, sufficient quantities of pure proteins were generated for three separate mice immunizations (spaced two weeks apart).

### Monoclonal antibodies

Fusing splenocytes to myeloma cells produced hybridoma colonies to be screened by IFA. Positive colonies secreted polyclonal antibodies and were therefore serially diluted, to isolate clones producing monoclonal antibodies. Further screening on CSFV-infected cells confirmed five clones producing anti-E2 mAbs, and two clones that secreted anti-Erns mAbs. Of the E2 clones, 60-127, 128, and 129 exhibited strong fluorescence (Figure 3.4a-c) than clones 54-21, 35 (Figure 3.4d-e). For Erns, clone 71-40 (Figure 3.4g) exhibited stronger fluorescence than 71-36 (Figure 3.4f). The mice immunization and hybridoma culture protocols successfully generated mAbs that can be used to detect CSFV Paderborn-infected samples.

### E2 Epitope mapping

E2 fragments were successfully cloned into pHUE vector, and then expressed and purified as described in the methods (Figure 3.5a). Western blots of these protein fragments

showed E2 mAbs showed that they all reacted to the N-terminal fragment (1-100 amino acids), suggesting that the epitope recognized is linear (Figure 3.5b and c, only 2 clones shown for reference). Further studies will go towards fine mapping the identified region to identify if complimentary mAb pairs can be employed in a blocking ELISA. We compared this 100-aa region to reference aa sequences from Genbank using CLC Main Workbench. As shown in Figure 3.6a, this region is well conserved between CSFV strains Paderborn (Genbank ascension number: AY027673.1) and Brescia (AAT85717), but is poorly conserved in BVDV1 (DQ088995.2) and BVDV2 (AF145971.1) sequences (Figure 3.6b). Therefore, the E2 monoclonal antibodies are good candidates for developing specific diagnostic assays.

## **Discussion**

In this study we generated and characterized a panel of Mab's against E2 and Erns, which are the most immunodominant CSFV proteins (Gavrilov et al., 2011). These proteins are also critical in virus attachment and entry into cells, and are important virulence determinants (Dräger et al, 2015; Leifer et al, 2013). The hybridoma clones we isolated produced mAbs that reacted strongly (as determined by fluorescence) on IFA with CSFV-infected cells. The E2 mAbs (especially 60-127,128, and 129) appeared to react with a greater percentage of infected cells compared to the Erns mAbs (Figure 3.4a-c). This is to be expected since E2 is the most abundant CSFV glycoprotein, so more E2 is available for antibody binding (Wensvoort et al., 1990; Wang et al., 2014). Based on their reactivity pattern on IFA, the E2 mAbs were chosen for further investigations. All mAbs mapped to the same 100 aa N-terminal region (Figure 3.5b and c), suggesting a limited variation between epitopes, possibly due to MHCII restriction. The N terminal region of E2, contains domain B/C, which has been shown to be highly immunogenic and is also poorly conserved from CSFV to BVDV (Figure 3.6) (van Rijn et al., 1996; Paton et

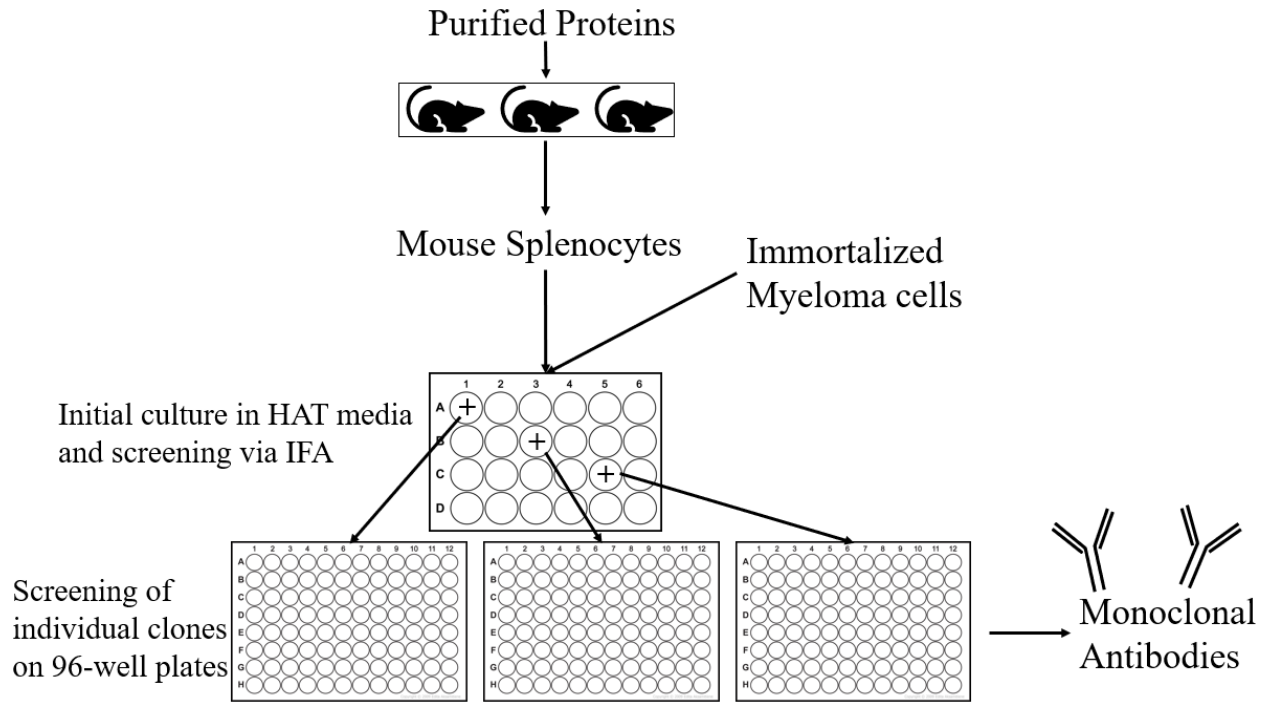
al., 2000; El Omari et al., 2013). The Paderborn strain that we used to generate the antigens belongs to genotype 2.1; therefore, our mAbs may have higher affinity to E2 and Erns sequences specific to this genotype. Fine mapping with smaller overlapping fragments is required to determine the exact epitope that these antibodies are recognizing. Moreover, we need to test the mAbs against strains from different genotypes and from different pestiviruses to assess their level of cross-reactivity. By mapping their binding epitopes, serological assays can be developed to detect a wide range of strains (conserved epitopes), or to distinguish between them (variable epitopes). The same should be done with the Erns mAbs. Recently Erns ELISAs have been developed to accompany E2 subunit vaccines and differentiate infected animals from vaccinated animals. However, these assays lack the sensitivity required for screening large populations of animals (Aebischer et al., 2013; Meyer et al., 2017). The generated mAbs should also be isotyped since certain IgG isotypes are more stable than others, and this is an important consideration when designing assays.

We want to use these proteins to construct an improved blocking ELISA that is highly specific to CSFV. This way it can be quickly employed in various surveillance systems without the need of sequential testing. Another promising serological assay is fluorescent microsphere immunoassay (FMIA), which relies on polystyrene/latex microbeads coupled to antigens or antibodies. Bead sets coupled to different antigens/antibodies can be analyzed in a single reaction using a specialized flow cytometer, allowing for extensive multiplexing (Krishnan et al., 2009). Xia et al. (2015) describe an FMIA for CSFV detection; it utilizes E2 and Erns antigens from both CSFV and BVDV to account for cross-reactivity. However, since this assay detects antibodies to E2/Erns, it will generate false negative results during early infections (before antibodies have developed), or in pigs that produce low levels of antibodies. By coupling the

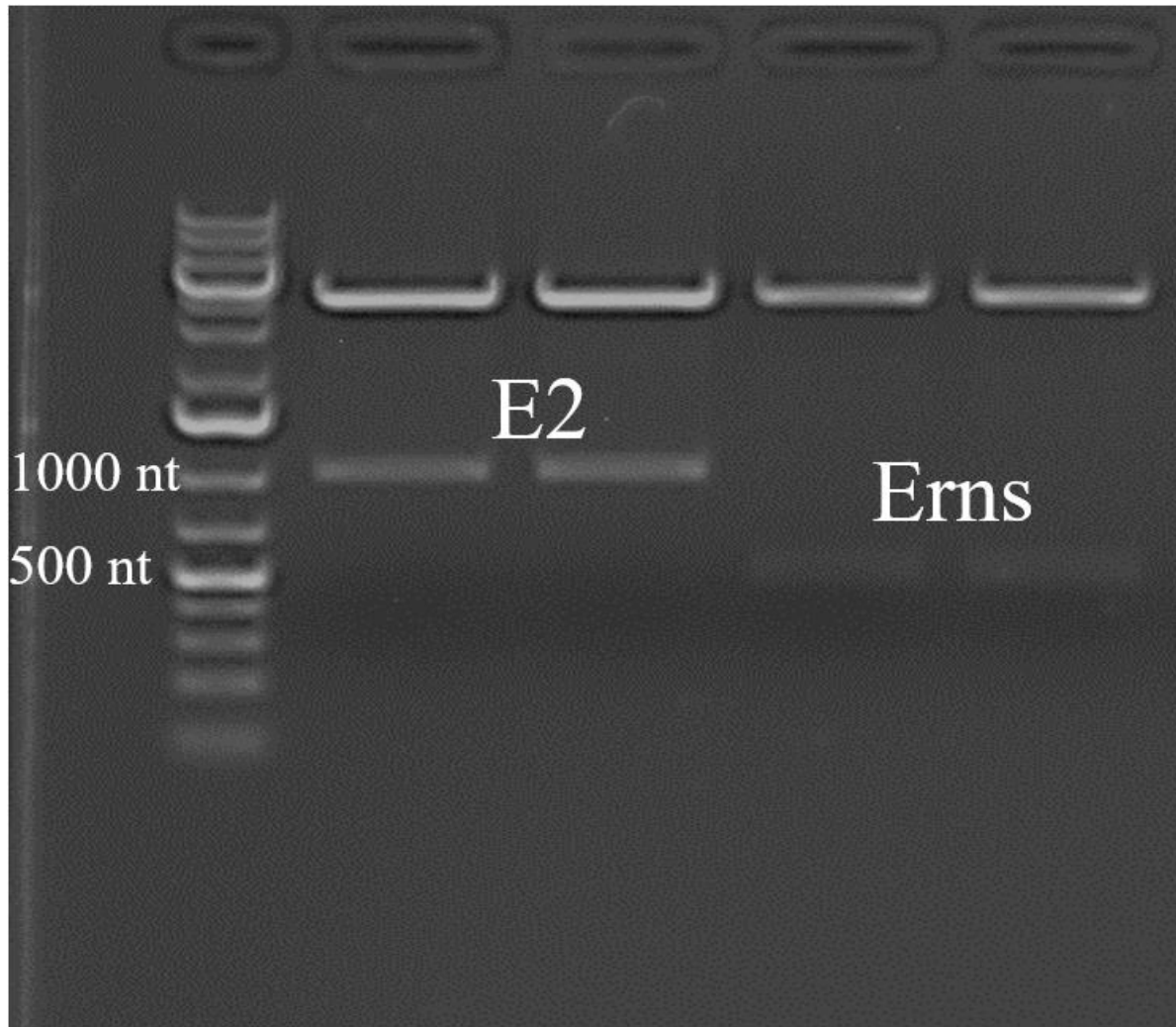


mAbs to beads we can capture antigens to detect infection sooner. Furthermore, this assay could be used to detect CSFV in oral fluids, which have low to undetectable levels of antibodies (see Chapter 2; Petrini et al., 2017). Both blocking ELISA and FMIA can be employed quickly and can be modified for multiplexing and to accommodate different sample types (i.e. oral fluids).

**Figure 3.1 Monoclonal antibody workflow**

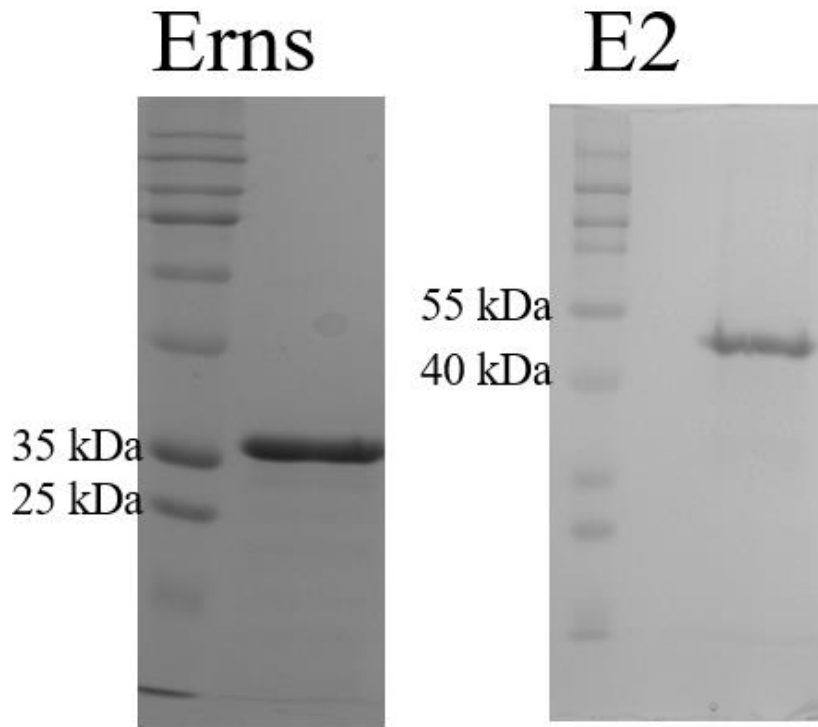


**Figure 3.2 Restriction digestion on agarose gel**



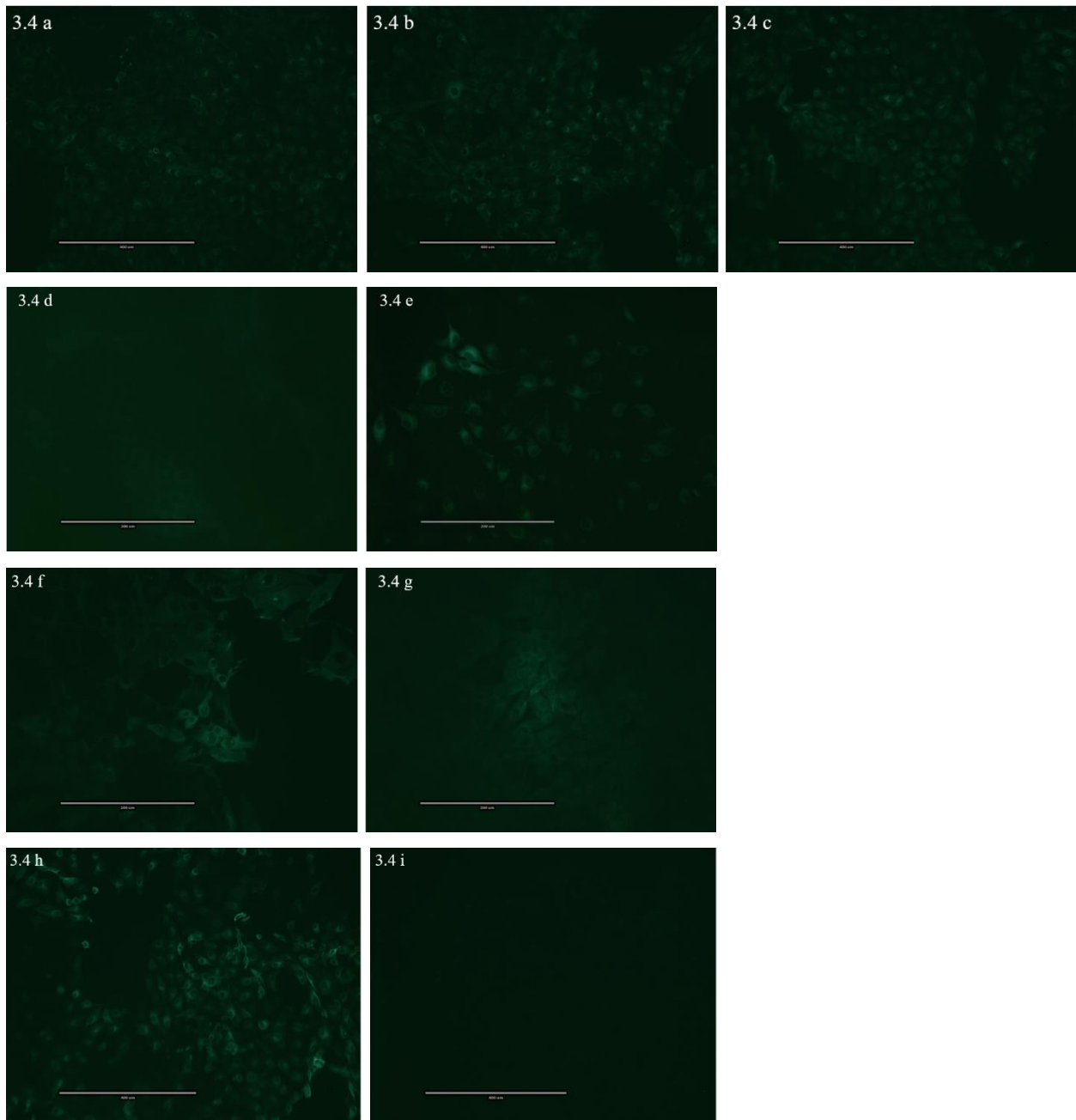
**Figure 3.2** Restriction digestion of E2 (lanes 2 and 3) and Erns (lanes 4 and 5) in pET28a vector, performed in duplicate. Performed on 1% Agarose gel.

**Figure 3.3 SDS-PAGE of protein fragments**



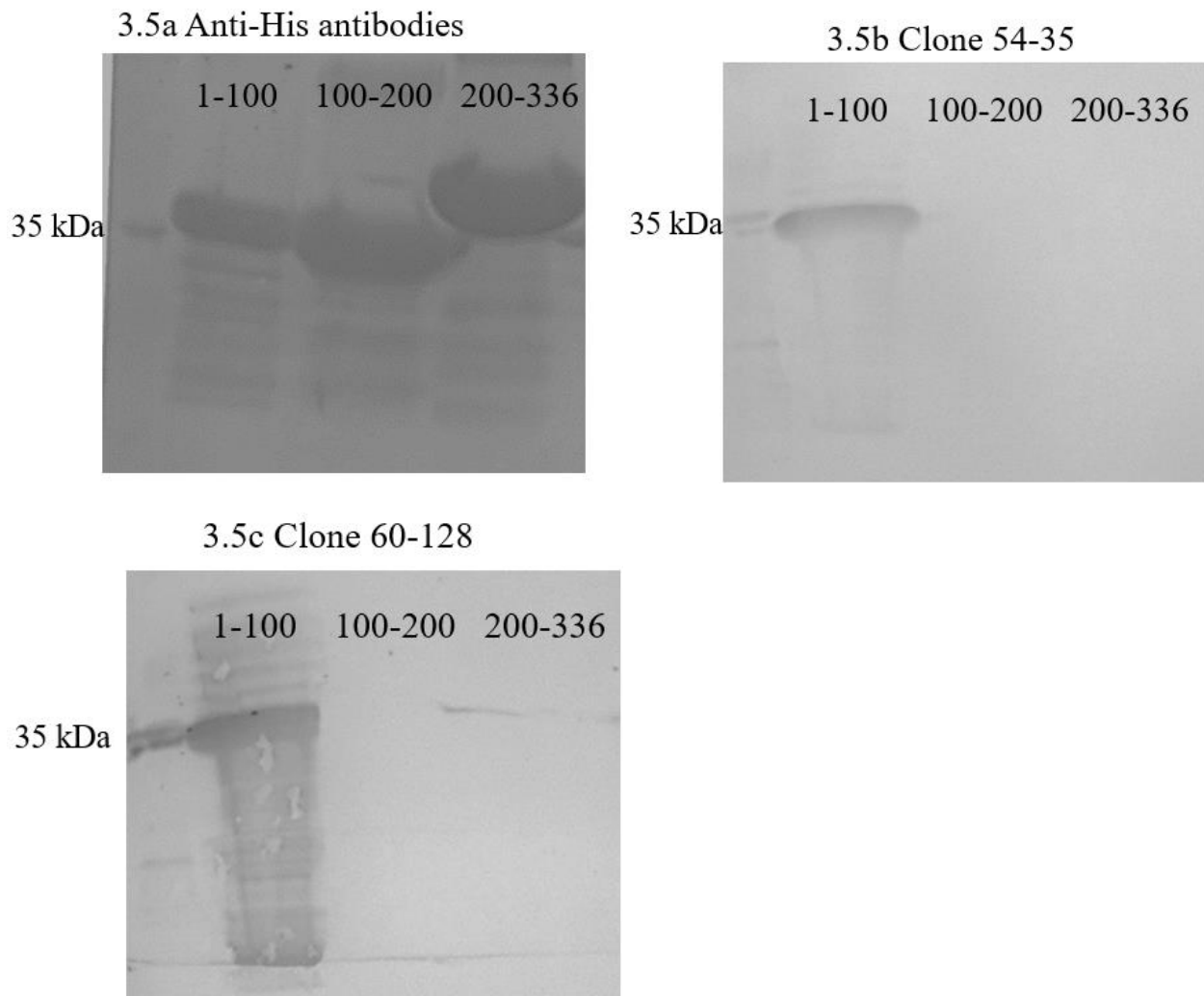
**Figure 3.3** SDS-PAGE (12.5%) gel of Erns and E2 confirming expression of correct size protein fragments.

**Figure 3.4 IFA screening of hybridoma clones**



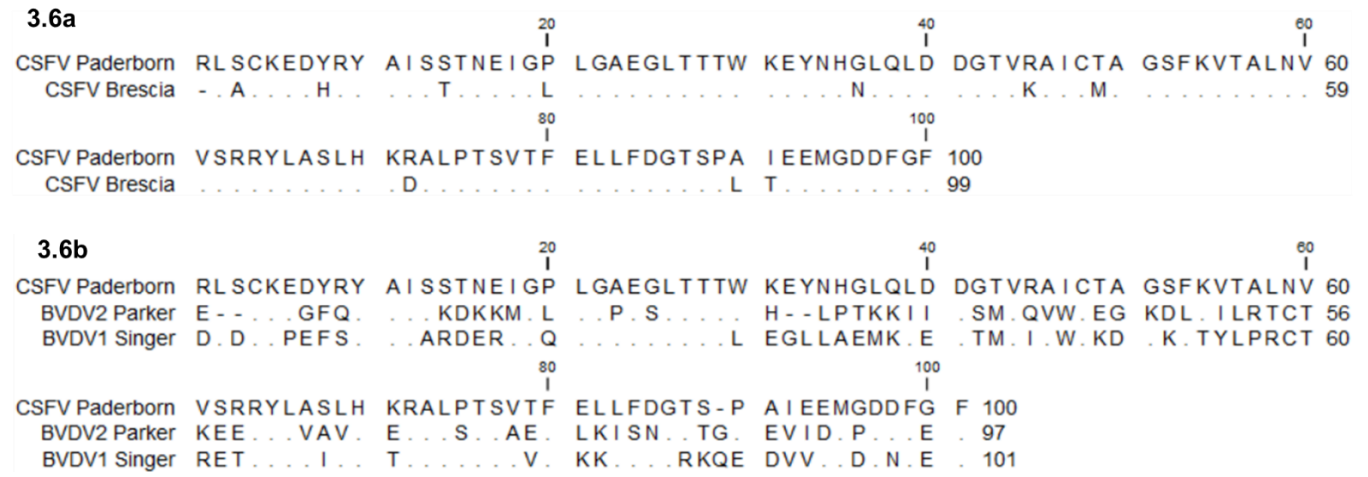
**Figure 3.4** IFA screening of hybridoma clones: (a-c) E2 clones 60-127, 60-128, and 60-129; (d-e) E2 clones 54-21 and 54-35; (f-g) Erns clones 71-36 and 71-40; (h-i) Positive control WH303, and uninfected negative control cells. Images are at 10X magnification for panels a, b, c, h, and i; images are at 20X magnification in panels e-g. Marker line measures 400  $\mu\text{m}$  and 200  $\mu\text{m}$  for 10X and 20X, respectively.

**Figure 3.5 Western Blot of E2 fragments**



**Figure 3.5** Western Blot of E2 fragments analyzed with (a) anti-His (aa 1-100, 100-200, 200-336 in lanes 1-3, respectively), (b) monoclonal antibody 54-35 (reacting to aa 1-100, lane 1), and (c) monoclonal antibody 60-128 (reacting to aa 1-100, lane 1)

**Figure 3.6 Sequence Alignment of Paderborn region 1-100 amino acids**



**Figure 3.6** Amino acid sequence alignment of CSFV Paderborn N-terminal region (1-100 aa; Genbank reference number: AY027673.1) with a) CSFV Brescia (AAT85717) and b) BVDV1 Singer (DQ088995.2) and BVDV2 Parker (AF145971.1).

## Chapter 4 - Conclusion

Classical swine fever has plagued swine and swine producers for over 180 years, and has been recorded on all inhabited continents throughout its history (Edwards et al., 2000). Today we understand that CSFV can cause a variety of disease presentations ranging from acute hemorrhagic fever to moderate chronic disease, to nonclinical infection (Moennig et al., 2003). Moreover, we have developed multiple vaccines to combat the virus, along with a large repertoire of diagnostic tools by which we can assess its virulence, pathogenesis, genotype, and geographic distribution (Blome et al., 2017). We still need a clear picture of what determines a strain's virulence and what host and virus factors dictate progression of one disease state versus another (Hulst et al., 2012). Regardless, scientific research along with strict stamping out campaigns have made it possible to eradicate the disease from multiple countries. Unfortunately, stamping out policies are not feasible in much of the developing world where CSFV persists. It is too expensive to compensate farmers for their lost pigs, and it would put extensive psychological and economic strains on small back-yard farms. Therefore, many countries must live with CSFV in back-yard farms and work to vaccinate more lucrative, industrial farms. Unfortunately, this allows the virus to persist in populations and mutate in response to vaccine pressure (Tang et al., 2008; Leifer et al., 2012; Pérez et al., 2012).

Due to this persistence throughout the world, countries that have eradicated the disease (i.e. the United States) must implement trade restrictions and surveillance programs to introduction of the virus. These efforts are complicated by the intensive swine production systems that house thousands of pigs together, usually limited to a specific geographic area within the country ("USDA - NASS, Census of Agriculture," 2012). Furthermore, the virus can



be easily transmissible in contaminated pork products, which could easily make their way into the US (Edwards, 2000; Casagrande, 2002; “Agriculture Fact Sheet | U.S. Customs and Border Protection,” n.d.). Infection of a large pig farm could go undetected and allow the virus to spread to multiple facilities before being detected. Such an outbreak would cause the death of thousands of animals and cause billions in lost production and trade (Meuwissen et al., 1999; Terpstra and de Smit, 2000). The current surveillance program is ill equipped to handle this scenario, due to its reliance on single animal sampling and screening tests which cannot reliably sample large swine farms (“Appendix B: Classical Swine Fever (CSF) Surveillance Plan,” 2007). These limited diagnostic techniques would also limit the effectiveness of eradication efforts if the virus were to become endemic in domestic or feral swine populations.

To help address these shortcomings we conducted studies on pooled oral fluid (OF) sampling in a CSFV-infected study group. Unlike serum or tissue sampling, OF samples can be collected with limited training and expertise and without handling and stressing the animals being sampled. Most importantly by hanging braided cotton ropes in a pig pen one can efficiently sample most of the pigs in that pen; whereas serum sampling can only feasibly sample a small subset of pigs from the entire farm (Prickett and Zimmerman, 2010). Rope sampling from OF has already shown to be effective at detecting porcine reproductive and respiratory syndrome virus (PRRS), porcine circovirus 2 (PCV2), swine influenza (SIV), African swine fever (ASF) and foot-and-mouth disease (FMD) (Prickett et al., 2008; Detmer et al., 2011; Prickett et al., 2011; Grau et al., 2015; Giménez-Lirola et al., 2016). Previous OF studies with CSFV showed that the virus could be detected using qRT-PCR in both individual animals and populations (Mouchantat et al., 2014; Grau et al., 2015; Dietze et al., 2017; Petrini et al., 2017). Our work builds upon these studies, since we utilized the Paderborn strain to induce a moderate

disease model. This model simulates the three different disease presentations encountered in the field: subacute, chronic, and recovered/persistent (Moennig et al., 2003; Uttenthal et al., 2003). We demonstrated that CSFV is detectable in OF at 8 days post infection (DPI) and be used to track the progression of virus in the population. Rope OF sampling allowed us to detect high virus titers in the study group even after most individuals had undetectable virus levels in serum. Most importantly we accomplished this with less effort and resources than for serum sampling. Future studies should attempt to validate these methods in larger populations in a field setting. Diagnostic assays optimized for OF should also be developed to improve the detection capabilities of this method even further. This work clearly demonstrates the potential of pooled OF sampling for CSFV surveillance.

To help improve screening techniques, we produced monoclonal antibodies (mAbs) specific to CSFV surface antigens, E2 and Erns. These antibodies are necessary for detecting CSFV in virus isolation and virus neutralization assays, as well as serological assays like enzyme-linked immunosorbent assays (ELISA) (“OIE Terrestrial Manual,” 2014). The latter have been used extensively to screen for CSFV, but positive results must be followed up with confirmatory tests due to the potential for cross-reactivity with other pestiviruses (i.e. bovine viral diarrhea virus) (de Smit et al., 2000; Dewulf et al., 2004). The antigens we utilized were based on the Paderborn reference genome; this strain belongs to a genotype that has an extensive geographic range (Beer et al., 2015). Immunization of mice with the purified antigens, along with hybridoma culture, yielded mAbs specific to E2 and Erns. More work must be done to characterize the specific binding epitope, isotype, and cross-reactivity of these mAbs. We plan to incorporate these mAbs to improve the specificity of blocking ELISA, which utilizes mAbs to block and compete with antibodies from test samples to limit non-specific binding (Wensvoort et

al., 1988). We would also like to incorporate the mAbs into a fluorescent microsphere immunoassay (FMIA), since this assay is efficient at multiplexing mAbs against multiple pathogens in a single reaction (Krishhan et al., 2009). Through multiplexing, CSFV testing can be incorporated into surveillance programs for common pathogens; this would maximize cost efficiency and active sampling for the virus.

To keep up with highly infectious foreign animal diseases like CSFV, it is necessary to continuously improve diagnostic methods. Taken together, OF sampling and specific mAbs are can improve surveillance efforts for CSFV. OF sampling facilitates surveillance of large pig populations, while utilizing limited resources compared to traditional serum sampling. Monoclonal antibodies can be utilized in a variety of serological assays that can detect CSFV quickly and specifically. Serological assay can be adapted to be used in the field and for multiplexing, which greatly maximizes the efficiency of these assays during surveillance efforts. This continued innovation helps to not only protect the US from CSFV introduction, but can be used in endemic regions to facilitate eradication of the disease worldwide.

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