PRODUCTION OF PORCINE SINGLE CHAIN VARIABLE FRAGMENT (SCFV)
SELECTED AGAINST A RECOMBINANT FRAGMENT OF PORCINE REPRODUCTIVE
AND RESPIRATORY SYNDROME VIRUS NON STRUCTURAL PROTEIN 2

by

TAMMY L. KOOPMAN

B.S.M.T., University of Texas at San Antonio, University of Texas Health Science Center at San
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Approved by;

Co-Major Professor
Carol Wyatt, PhD

Approved by;

Co- Major Professor
Richard 'Dick' Hesse, PhD

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Abstract

Over the last two decades molecular laboratory techniques have enabled researchers to investigate the infection, replication and pathogenesis of viral disease. In the early eighties, Dr. George Smith developed a unique system of molecular selection. He showed that the fd bacteriophage genome could be manipulated to carry a sequence of DNA coding for a protein not contained in the phage genome. Infection of the recombinant bacteriophage or phagemid into a specific strain of the bacterium, *Escherichia coli*, produced progeny phage with the coded protein displayed as a fusion with the phage's coat protein. Antibody phage display utilizes the same technology with the DNA encoding an antibody fragment. The DNA insert can carry the information to produce either a single chain variable fragment (scFv) producing the heavy chain variable and light chain variable (V_H-$V_{L)}$ portion or a Fab fragment which also contains the heavy chain constant 1 with the light chain constant (C_H and $C_{L)}$ portion of an antibody. Screening an antibody phage display library has the possibility of producing an antibody not produced in the normal course of immune selection. This decade also saw the emergence of a viral disease affecting the porcine population. The Porcine Reproductive and Respiratory Syndrome virus (PRRSV) has been one of the most costly diseases affecting the pig producer. Molecular investigations found that PRRSV is a single, positive-stranded RNA virus which codes for five structural and 12-13 nonstructural proteins producing an enveloped, icosahedral virus. An interesting characteristic of PRRSV is the ability to produce infective progeny with genomic deletions, insertions and mutations within the nonstructural protein 2 (nsp2). With this knowledge, many researchers have produced marker vaccines containing fluorescent tags with the hope of developing a DIVA (Differentiate Infected from Vaccinated Animals) vaccine. In my Master's studies, I studied the techniques of antibody phage display technology and how to apply these methods to producing scFvs which recognize a recombinant PRRSV nsp2 fragment protein and the native protein during infection of MARC-145 cells.

Table of Contents

List of Figures

List of Tables

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Dedication

The following is dedicated to my family – the love of my life, Francis and my wonderful children, Sean, Madison, Kailey, and Ethan Koopman, for their love and understanding during the last few years.

Chapter 1 - Literature Review and Significance

Porcine Reproductive and Respiratory Syndrome Virus

Historical Overview of Emergence

At the end of the 1980s, an epidemic occurred in North Carolina among pig herds. The causative agent was unknown and the clinical signs of infection were called "Mystery Swine Disease".[2,3] Producers reported severe reproductive losses due to abortion storms amongst their sows, post weaning pneumonia and decrease in performance related to growth, even death among the piglets. Several known porcine pathogens were suggested as the etiological agent - viruses and bacteria such as classical swine fever virus, porcine parvovirus, Pseudorabies virus, porcine enterovirus, *Leptospira interrogans* serovar *bratislava*, *Chlamydia psittaci*. Isolation of the causative agent was difficult, because pigs showing signs of infection often were infected with these pathogens. A spread of disease was quickly seen in the U.S affecting herds in other pork producing areas such as Minnesota and Iowa. Disease was seen in Japan in 1988, and quickly spread throughout neighboring countries. November 1990, Germany reported outbreaks with clinical signs similar to disease seen in the U. S. By the summer of 1991, outbreaks of infection were seen in the Netherlands, Belgium, Spain and Great Britain. By the mid-1990s the pandemic had spread to most of the major swine producing areas in the world.[2-4]. The economic loss due to PRRS viral infection makes this virus one of the most expensive infectious diseases to pork producers throughout the world.

The clinical disease had several names - Mystery Swine Disease, Blue ear disease, Swine infertility and respiratory syndrome (SIRS) and Porcine Reproductive and Respiratory Syndrome (PRRS), the use of which depended upon the geographical area of the outbreak.[4,5] An international symposium held in Minnesota officially named the infection – Porcine Reproductive and Respiratory Syndrome. The causative virus would carry the same name.[4] The definitive etiological agent was resolved in Europe by the isolation of an enveloped, positive sense, single stranded RNA virus which fulfilled Koch's postulates.[6,7] Isolation of virus from a Minnesota herd experiencing clinical symptoms and subsequent experimental infection with tissue homogenates resulting in transmission of disease occurred that same year.[3,5,8] However, there is evidence of infection occurring in swine herds prior to the presentation of clinical disease. PRRSV specific antibodies were detected in sera as early as 1979 in retrospective studies of Canadian herds, but not until after 1985 in herds of the U.S. states of

North Carolina, Iowa and Minnesota. Retrospective studies of European and Asian herds mimic that of the U.S, with serological evidence in the mid-1980s.[3,9] There are two genetically distinct PRRS viruses, the European Lelystad virus (Type I PRRSV) and the North American ATCC VR-2332 (Type II PRRSV), which diverged from a common ancestor.[5,10,11] However, the two different types share genomic organization and similar pathogenesis. Both viruses are genetically diverse, which have led to further divisions within the European genotype.[11,12] The two different strains may have a common ancestor which originated in wild boars of Eastern Europe and possible spread to the US through the transfer of these animals in 1912. Ironically the two strains emerged as a disease causing virus around the same time.[13-15] Emergence of disease was facilitated by the changes of swine husbandry within the last century. Swine production changed from relatively small farms to large herds, movement and transport of pigs worldwide, increased use of breeding programs, and replacement of animals from fewer but larger companies.[3,5]

Structural Biology of PRRSV

The 10th International Congress of Virology held in Jerusalem in 1996, established the new family of enveloped positive strand RNA virus, the *Arteriviridae*, which contained Equine Arteritis Virus (EAV), Lactate Dehydrogenase Elevating Virus (LDV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Simian Hemorrhagic Fever Virus SHFV.[16] A new order classification, *Nidovirales*, was established comprising of families of *Arteriviridae* and *Coronaviradae*. These four viruses were group together because of their molecular and biological properties. They cause a wide range of disease symptoms, from relatively asymptomatic (LDV) to death (SHFV, PRRSV). All have a fairly restrictive host range. EAV disease has been studied since the late 19th century with LDV and SHFV investigations starting in the 1950s. PRRSV emerged within the last twenty years. EAV and PRRS have several similarities, both are transmitted through the respiratory route, cause abortion, the virus persistence in semen and severity of disease is strain dependent.[16] The two genetically distinct PRRS viruses, the European Lelystad virus (Type I PRRSV) and the North American ATCC VR-2332 (Type II PRRSV) share genomic organization and similar pathogenesis.[5,10,11] Both viruses are genetically diverse with in the two strains and further divisions within the European genotype have been classified.[11,12] Host cell restrictiveness of the arteriviruses makes propagation in culture difficult. Macrophages are the primary target cell for virus infection and replication; however, transfecting the genome into other non-permissive cell lines causes infection.[16] Porcine alveolar macrophages (PAMs) and blood monocytes are the only porcine cells that can be used to successfully produce PRRSV in culture.[11,16] However, PRRSV RNAs and antigens have been identified in other cells and

2

tissues in naturally infected pigs such as Peyers patches, kidney, lung, lymph nodes. In experimentally infected pigs the evidence of virus infection was also found in heart, germ cells, and epithelial-like cells.[11] Permissive non-porcine cell lines that have been used extensively in PRRSV experimentation and propagation for both wild type and infectious clone studies is the MARC-145 cell line, a subclone of the MA104 monkey kidney cells, and the CL2621 cells.[11,17,18] Virus infection in the MARC-145 cells has two phases, a primary and secondary infection. The primary infection phase is 20-22 hours post inoculation of the virus in the MARC-145 cell culture and the secondary infection phase of progeny virus is two to three day post inoculation. Cytopathic effect (CPE) is seen in the secondary phase of infection.[11] In natural infection the progeny virus is a quasi-species of related genotypes with a high degree of variability.[19]

The PRRS virus is spherical with a diameter of 60nm and structurally resembles coronaviruses.[11,20] The icosahedral nucleocapsid is composed of a single protein and is surrounded by a smooth, lipid envelope containing five structural proteins - the N, M, GP_{2a}, GP_3, and GP_4, GP_5. The N, M, and GP_5 proteins represent 90-95% of the structural proteins in the virus particle. These major structural proteins are essential for particle formation. The minor glycoproteins are GP_{2a}, GP_{2b}, the soluble GP_3, and the membrane-bound GP_4; which are translated from the ORF2a, 3 and 4 respectively and are essential for virus infectivity.[21,22] As seen in EAV, incorporation of the four proteins in the virion envelope is dependent on the formation of their multimeric complex.[21,23] The monomer GP_{2a} is needed for virus replication and is retained in the ER. Embedded within the ORF_{2a} is the sequence encoding the ORF_{2b} E protein.[11]

The phosphorylated N protein exists as a homodimer linked by disulfide bonds and encoded by the ORF7 PRRSV gene.[11,16] A characteristic shared by *nidoviruses* is the phosphorylations of their nucleocapsid proteins, which provides not only a structural component but non-structural factors as well, and are related to virus pathogenesis.[11] PRRSV infected pigs contain a high concentration of antibodies specific to the N protein. There are five conserved antigenic domains in the N protein of the NA VR-2332 strain and four for the EU LV strain. Antibodies directed against these epitopes do not prevent PRRSV infection of cells. Three of the epitopes are linear with the remaining 2 being discontinuous conformational sites. Covalent and non-covalent interactions are mediated by three conserved cysteines of the N protein located at position 23, 75, and 90. The cysteine at position 23 is responsible for the disulfide bonds between the viral N proteins which when disrupted prevent infection. Interestingly the cysteine 90 is absent from the EU PRRSV isolates; however when disrupted in the NA PRRSV infectious clone renders the virus non infective.[11,24] The N protein contains two nuclear localization sequence signals (NLS) which is responsible for the translocation and accumulation of the

protein in the nucleolus and is independent of other viral proteins. Virulence and pathogenesis is associated with this localization function of the N protein, modification of the NLS site causes an attenuated virus infection.[11] Interaction with other host proteins, such as fibrillarin, suggests the N protein could regulate host gene expression.[25]

The conserved non-glycosylated M protein is encoded by the ORF6 viral gene and functions in virus assembly and budding. The ORF5 viral gene, glycoprotein GP_5 forms disulfide-linked heterodimers with the M protein in the endoplasmic reticulum of the host cell where it has accumulated.[26] The bonding of the two proteins is essential to virus assembly; mutation of key cysteines in either protein prevents virion assembly. Virus attachment to host macrophages sialoadhesin by the GP_5-M protein complex has been proposed because of its interaction with heparin.[27,28] The GP_5 protein is expressed on the surface of the virion and GP_5 specific antibodies are shown to neutralize virus *in vitro*. An epitope located in the middle of the ectodomain of the GP5 is the main neutralizing epitope.[29] There are three areas of the PRRSV genome that have a high degree of variability, the ORF5 gene being one area along with the ORF3 gene and nsp2. The high gene variability translated into the GP5 protein has made vaccine development using the GP5 protein as the target difficult. [30,31] The GP5 N-glycoslyation sites are instrumental to virion infectivity, immune evasion, and virus persistence – there are three such sites and in some NA strains four sites. Protein conformational structure and functional activity relies upon N-linked glycosylation. N-glycan neutralization shielding is a mechanism that shields the neutralizing epitopes reducing the immunogenicity of the site. GP_5 was shown to induce apoptosis in infected PAM and MA-104 cells. Apoptosis is seen in non-infected adjacent cells suggesting the release of GP_5 from the infected cells.[32]

The minor structural proteins are encoded by the ORF2, 3 and 4 genes. The three proteins are linked by disulphide bonds in a heterotrimer located in the envelope of the PRRSV virion. The formation of the heterotrimer is essential to virus infectivity. ORF2 gene encodes three different proteins – the N-glycosylated GP_{2a}, the non-glycosylated E protein, and the embedded GP_{2b} protein. The GP2a protein is not essential for virus particle formation; however, deletion of the ORF2 and 4 genes does not produce viable virus in culture.[33] All arteriviruses contain an E protein, which is hydrophobic with a hydrophilic C terminus and is incorporated into the envelope membrane. The non-covalent association of the E protein with the N protein stabilizes the virion core and is required for viral replication.[24] Bacterial cells expressing E protein had increased permeability of hydromycin B suggesting the protein may function as an ion channel and facilitate viral uncoating.[34] The ORF3 gene encodes the highly variable glycosylated GP_3 protein. The protein is a non-structural, soluble glycoprotein for LDV, a structural envelope protein for EAV, and both structural and soluble for the

PRRSV.[11] Interestingly, glycoslyation sites of the GP$_3$ protein are conserved in NA and EU PRRSV strains.[35] PRRSV infected pigs have low levels of anti-GP$_3$ antibodies, the protein is immunogenic. These antibodies may work to clear virus infection. The ORF4 encoded GP$_4$ protein was shown to be essential for virus replication and alters the transportation of viral and cellular proteins to the cell surface.[11]

PRRSV Genome Organization and the Nonstructural Proteins

The PRRSV is a positive sense, enveloped, single stranded RNA virus. Its genome is approximately 15.2 kilobases (kb) with a polyadenylated 3'end and a type I 5'cap structure. The genome is polycistronic, two open reading frames (ORFs) encoding the nonstructural proteins upstream from nine genes encoding mostly structural genes from subgenomic mRNA and flanked by 5' and 3' non-translated regions (NTRs). Because the PRRSV is a positive sense RNA virus, it functions as the genomic nucleic acid and as translatable mRNA; therefore, the genomic ssRNA can be replicated, transcribed, or translated. Nested subgenomic mRNA is a characteristic of all nidoviruses - nido from "nidus" meaning nest in Latin. Three quarters of the genome is occupied by the two replicase ORFs – 1a and 1ab. ORF1a is variable in length whereas ORF1ab is more conserved. The 5' common leader sequence contains short ORFs which are on all the translated mRNAs and are thought to be a translation rate regulator of the subgenomic mRNAs. Discontinuous transcription of negative strands produces subgenomic mRNAs containing the genomic sequences of the 3' and 5' NTR. A conserved junction site (JS) is located at the end of the 3' leader sequence and between the mRNA body and the end of the 5'NTR. The genome contains 17 other JS sequences. Two polyproteins are derived from the replicase genes – ORF1a and ORF1ab proteins. The ORF1ab polyprotein is cleaved by three proteases encoded by ORF1a into 12 nonstructural proteins (1-12 nsps) possibly13. The ORF1a encodes the polyprotein 1a (pp1a), which is cleaved into nine nonstructural proteins – nsp1α, nsp1β, and nsp2-8. The pp1a cleavage products function as proteases to process the pp1a and pp1ab nsps. These nsps function in virus replication and transcription.[11] The nsp1, 2, and 4 proteases are conserved among arteriviruses, due to their proteolytic function are possibly toxic to cells.[36,37] A cysteine autoprotease domain within the nsp1 and nsp2 rapidly releases them from the polyprotein. The 3C-like serine protease (SP) nsp4 cleaves sites containing the dipeptide (Glysine/Glutamine)-(Glysine/Serine) with the same specificity as 3C-like cysteine proteases, eight conserved arteriviruses' cleavage sites have been identified, five in ORF1a and three in ORF1ab. The ORF1a polyprotein also contains a papain-like cysteine protease located at the N terminus, PCPα. The 3CLpro cleaves the remainder nsps from the polyproteins. In EAV, nsp1 was shown to function in polyprotein 1ab processing, replication and

5

transcription. The nsp9 contains the RNA-dependent RNA polymerase motifs; whereas the nsp10 contains a RNA helicase with metal binding properties is critical for subgenomic mRNA transcription. The nsp10 protein is thermolabile and has NTPase activity which is pH sensitive.[38]

Non-structural Protein 2

The nsp2 is the largest of all the nsps and contains the most genetic differences between the two PRRSV genotypes (NA and EU strains).[39,40] The protein contains approximately 1100 amino acids. It contains three domains; an N-terminal cysteine proteinase domain (PL2), a middle region, and a hydrophobic transmembrane region. Natural mutations, insertions and deletions are seen in the middle domain, while the two remaining domains are conserved among field strains.[40] The fact that this variable middle region of the genome can withstand the deletions or insertions without loss of infectivity and proliferation has had researchers focus for several years as a major site for vaccine development.[30,41,42] The middle domain contains two areas of hypervariability (HV-1 and HV-2) flanked by conserved sequences of amino acids. *Han et al.* demonstrated with reverse genetics that deletion of several areas of the nsp2 protein were nonessential, as many as 309aa deletion of this domain did not prevent virus replication. But deletions encompassing the PL2 and transmembrane regions showed no viral growth and therefore were determined to be critical. Even though the deletion mutants produced resulted in viral growth, the growth kinetics were altered, they were slower to reach peak titer and had decreased cytolytic activity with delayed or absent CPE.[40] This area of the nsp2 may not be essential for infectivity and growth but it may have a great deal to do with virus virulence and fitness. The PL2 (cysteine protease domain) has cleavage activity in both *cis* and *trans*.[37,43] The nsp2 of EAV has been shown to generate the double membrane vesicles with nsp3 and perform co-factor functions with nsp4 to cleave other viral products.

Several immunodominant epitopes of the PRRSV nsp2 protein have been identified by Pepscan technology for the Type II strain. The areas identified were shown to be in predicted hydrophilic areas of the protein. Antibody recognition of peptides by convalescent PRRSV infected pig sera identified 18 such areas.[44] Another study utilizing a phage display library of Type I PRRSV peptides and experimentally infected pig sera identified six linear B cell epitopes. One site, ES7, showed positive antibody binding of sera early from most of the infected pigs post infection, with all pigs eventually producing antibodies specific to the site[45].

The 2007 emergence of a highly pathogenic strain of PRRSV in China, which contained a thirty amino acid deletion within the middle domain, suggest the immunological importance of the nsp2

protein.[46,47] The discontinuous deletion was partially located in the hypervariable region 1 (HV-1). Initially this deletion was thought to be the cause of the increase of virulence in these strains of virus; however recent research has disputed this hypothesis. HV-1 deletion mutants did not produce progeny virus in MARC-145 culture cells.[48] B cell epitopes predicted by Pepscan technology and the subsequent production of synthetic peptides to hydrophilic amino acid sequences reacted with sera collected from experimentally infected piglets identified four epitope sites. Comparison of amino acid sequences of the four epitope sites with other Type II NA strains found they were relatively conserved.[49]

PRRSV Infection and Pathogenesis

The PRRSV can survive outside a host cell for extended periods of time in temperatures lower than 20^0C and as low as -70^0C. A pH above 7.5 or below 6.5 decreases the stability of the virus and lowers its infectivity. The virion is sensitive to solvents and detergents which are detrimental to the envelope, rendering the virion non-infectious.[11]

Entry of the PRRSV into a permissive cell is receptor mediated. Heparin sulfate, vimentin, and sialoadhesin have been shown to bind and internalize virus when these receptor are expressed in non-permissive cell lines. However, entry into the cell by these receptors alone does not produce progeny virus suggesting the requirement for other factors. *Lopez-Fuertes et al.* identified the monocytic receptor CD163 along with sialoadhesin as the cellular proteins needed for PRRSV to attach and enter primary pig macrophages.[28,50] Expressing these two proteins in non-permissive cell lines produces progeny virus when infected with PRRSV. [51] CD163 interacts with the viral coat proteins GP_{2a} and GP_4 to facilitate entry into cells. CD151, a cell signaling and activation protein, binds the 3' UTR of PRRSV RNA and is critical to MARC-145 infection.[11,32] Anti-CD151 antibodies added to cultured MARC-145 cells blocks the PRRSV infection.[32] To infect cells, PRRSV requires a low pH of intracellular membranes. Replication of the virus occurs within the cytoplasm.[11,17] Upon infection the replicase protein is expressed by translation of the ORF1a and ORF1ab genomic ssRNA.[40] A ribosomal frameshift of -1 occurs between the translation of ORF1a and ORF1b. A "slippery sequence" occurs in the frameshift area, along with a pseudoknot structure which causes the shift in translation. In EAV, *den Boon et al.* showed that the efficiency of the frameshift was 15-20%. The first nsp to be processed is nsp1 with the papain-like cysteine proteases (PCPα and PCPβ), which are contained within the nsp1 protein. In EAV, nsp1 was shown to function in polyprotein 1ab processing, replication and transcription. Inactivation of PCPα by mutation prevented replication of subgenomic mRNA and the production of structural proteins; however, genomic replication was not affected. The inactivation of

PCPβ prevented virus replication and RNA synthesis. The two proteases are essential to process the nsp1α and nsp1β and thereby genome replication.[11] The 3CLpro proteinase, located in nsp4, cleaves the remainder nsps from the polyproteins. The cysteine proteinase (PL2pro) in the N terminus of the nsp2 cleaves at the nsp2-nsp3 junction.[52] During replication the virus forms paired and double membrane vesicles (DMVs) within three hours of infecting the cell. A complex scaffolding forms by the non-covalent interaction of the C-terminal end of nsp2 to nsp3 which helps to form the DMVs.[11,16] These structures are membrane bound and function as the site for viral RNA synthesis.[16,40] The DMVs and nonstructural proteins localize to the perinuclear region of the infected cell.

Replicative intermediates (RI) are negative strand copies of the subgenomic mRNAs involved in synthesis of more positive strand mRNAs. The subgenomic mRNAs contain two separate areas of the genome sequence, the 3'end and one third of the 5' terminal end. The junction site (JS) sequence links the two areas together. PRRSV genome contains several of these JS boxes and the sequence of each are not conserved. Variation in the JS sequences suggests that it may be derived from either the leader or body of the genome sequence.[16]

Infection of cellular culture is cytocidal. The severity of clinical signs is variable and affects different pig breeds equally.[4] Infected PRRSV pigs show a robust immune response; however, persistent infection with prolonged viremia and possible reinfection has been detected.[36] The appearance of neutralizing antibody is variable, appearing weeks after infection or not at all.[12] An immune dysregulation is seen in experimentally infected isolator piglets. Increased hyperplasia of the lymph system is observed with hypergammaglobulinemia of greater than 10 fold compared to other porcine viral infections, and an occurrence of autoimmunity.[53] *Butler et al.* showed in PRRSV infection, the B cell clones being expanded exhibited an increase in hydrophobic antigen binding sites with a predominant IgM population, which is similar to a nonimmune B cell repertoire. These cells were also shown to express the CD2 cell surface protein which is seen in undifferentiated B cells.[54] Recombinant nsp1, nsp2, nsp7 and N proteins show that sera of infected pigs contain specific antibodies to each of these proteins.[55] The immune response has shown to be greater to the nonstructural proteins than to the structural N protein. The nonstructural proteins down regulate the immune response by inhibiting the expression of interferon (IFN) and the double stranded RNA signaling pathways.[56,57] The ability to have an effect on the immune response of infected animals may be responsible for the delay or inability to clear the virus.[11]

PRRSV Diagnostics and Control

Identification of PRRSV positive herds is essential to control of infection. The most common diagnostic test is the enzyme linked immunosorbent assay (ELISA) of the PRRSV N protein, commercially produced by Idexx laboratories, HerdChek PRRS 2XR. Falsely classifying a PRRSV negative herd as positive or a positive herd as negative due to test failures is a potentially costly problem. Confirmatory tests such as immunoflourescent assays (IFA) or PCR may be more specific but more costly as well.[58] The emergence of Type I like PRRSV strains in the US poses a problem of identification of those strains that will test negative with the current diagnostics.[30,59]

The current vaccination strategies include a modified live and killed PRRSV vaccine. Immunity to heterologous strains of the vaccine is effective; however, field strains of the virus are highly diverse and the vaccine is not always protective. Modified live vaccines pose a potential problem of reversion of virulence. Killed viral vaccines are not as effective in eliciting an immune response in pigs especially in the Th2 response.[60,61] The ability to differentiate infected from vaccinated is not possible with the current vaccines. Marker vaccines or DIVA – Differentiating Infected from Vaccinated – strategies would determine the immune status of the herd and facilitate the producer in movement of individual pigs from farm to farm or within the herd.

Phage Display Technology

M13 Bacteriophage Biology

One of the most abundant viruses on earth is bacteriophages. They are small viruses that infect bacteria. The M13 is an Ff class filamentous phage that infects the gram negative bacterium, *Escherichia coli*.[62] Infection of the bacteria is not lytic – does not cause cellular death. The *E. coli* continue to proliferate; however, at a decreased rate.[63] Filamentous phage (genus Inovirus) is composed of single stranded DNA genome encased by five different coat proteins.[64] There are nine open reading frames encoding eleven proteins, six functional and five structural.[63]

The structural proteins are g3p, g6p, g7p, g8p and g9p. The two ends of the phage, designated as either the A (proximal) or B (distal) end, contains different proteins and are referred to as the minor coat proteins. The A-end is composed of the minor coat proteins g3p and g6p and functions during infection and termination of phage assembly. This end is the first to enter the bacterium during infection, with about five copies of the proteins.[63] Proteins g7p and g9p compose the B-end and function in phage assembly.[64] It is the first end assembled in progeny virus, absence of either protein decreases the amount of progeny virus produced.[65] Assumptions based on symmetry, determined that there are

three to four copies of these distal end proteins. However, scientific evidence has not supported this assumption. The two proteins are difficult to purify and investigate their structure and function due to the low concentration. The g9p closely associates with the packaging signal.[63] The proximal end proteins, g3p and g6p, are instrumental to infection. The g3p with its tripartite structure binds the F pilus of the *E. coli*.[66] The major coat protein, g8p, is the most abundant protein with 2700 to 3000 copies arranged helically around the ssDNA producing a flexible outer sheath.[64] The length of the ssDNA determines the number of g8p copies, which assembles in a fish scale, helical fashion around the ssDNA. The g8p is a multidomain protein when inserted into the bacterial membrane prior to incorporation into progeny phage.[63]

The genome is organized into three functional clusters of ten tightly packed genes. The first cluster contains the genes 3, 6, 7, 8, 9; the second, genes 1 and 4; the third, genes 2, 5, 10. There are two non-coding intragenic regions located between genes 2 and 4, and between genes 3 and 8 with approximately 500 and 50 base pairs respectively. The larger intragenic region (IG) functions in the replication process of the bacteriophage; contains the origin of replication for both primary and complimentary strand synthesis, the morphogenetic signal (M), the termination signal, and an enhancer and promoter for gene expression. The smaller IG contains the promoter for expression of genes 2, 6, 1 and 6.[64] There exists a hairpin structure of 32 bp in the ssDNA at the distal end. It contains the packaging signal which functions to encase the ssDNA into progeny virus and determines the orientation of the strand within the g8p tube.[63]

Gene expression is driven by the eight to nine promoters and three terminators. Half of the genes are transcribed more frequently than the other half. Transcription is initiated at three different promoters and end at the same terminator, resulting in a higher concentration of some proteins over others. Post translational cleavage occurs in the larger transcripts by host enzymes. The functional proteins are g1p, g2p, g4p, g5p, g10p, g11p and function in replication, assembly and secretion of progeny virus. Functional transcripts vary with time; the smaller mRNAs (5, 7, 8, and 9) are longer lived with the larger (2 and 10) degrading within minutes. Translation of the mRNAs is regulated by the ribosome binding sites and g5p (ssDNA binding protein). The g5p binds to the translational operator of various phage mRNAs located in the transcripts' 5' leader sequence and terminates transcription. The ssDNA genome is orientated so that the genes coding for the g8p is located at the A-end and a non-coding intragenic region is located at the B-end of the phage.[67]

The M13 bacteriophage infects by attaching of the minor coat protein g3p to the F- pilus and the co-receptor TolA of the *E. coli* bacterium.[67,68] The pilus is cell surface receptors encoded by the male *E. coli*.[64] The number of pili on the surface of the *E. coli* is relatively low and are continuously

expressed and removed from the surface of the cell [68] The g3p is tripartite, domains N-terminus 1 (N1), N-terminus 2 (N2) and the C-terminus (CT) separated by glycine linkers.[66] The N2 binds the pili enabling the N1 domain to bind the TolA protein [68]. Retraction of the pilus initiates disassembly of the phage by Tol proteins A, R and Q and the genome transported into the cytoplasm [67,68]

Replication of the phage ssDNA is accomplished by the rolling circle mechanism into a closed double stranded replicative form by host enzymes and occurs within 10 minutes of infection. Translation of the replicative form (RF) yields the ten specific phage proteins. Two of the viral proteins have important functional roles during replication. The g2p nicks the positive viral strand origin creating a free 3'-OH end which functions as a primer for rolling circle replication to begin. The g5p acts as a non-sequence specific ssDNA binding protein binding the synthesized phage genome and prevents formation of the RF. The ssDNA will then be available to be assembled into progeny phage. However, g5p must reach a threshold of concentration before packaging occurs.[67] As the structural proteins are produced they are stored in the inner membrane of the bacterium. Unlike most bacterial viruses, which assemble in the cytoplasm of the host cell, bacteriophage are constructed in or on the membrane [63] Assembly of the phage proteins into progeny virus is dependent upon three proteins; g1p and g4p and host thioredoxin. Homomultimers of the g4p produces a channel in the outer membrane of the *E. coli* and causes a conformational change in g1p which can then associate with the g4p channel and thioredoxin. The g7p and g9p are thought to interact with the packaging signal, they are the first two proteins to be assembled and secreted. The ssDNA of the phage is chaperoned into the phage assembly process by the g5p-ssDNA complex where the g5p is released.[64] The g8p replaces the g5p by wrapping around the ssDNA. Lastly, the attachment of g3p and g6p ends the assembly process.[63] The infected *E. coli* continues to replicate and divide while producing approximately 100 new viruses with each generation.

Phage Display

Bacteriophage research ushered in the science of molecular manipulation. The small size, easy isolation of phage DNA and proteins, and the reproduction cycle enabled the genetic and biochemical investigation of molecular mechanism.[63] Although the T4 and lambda bacteriophages have been used in the past experimental studies, the most common used today is the filamentous phage.[66]

Phage Display, a molecular technique where gene fragments, encoding millions of variants of certain ligands (peptides, proteins, enzymes, etc.), is inserted into the bacteriophage genome or as a phagemid vector, to produce a fusion product associated with one of the phage coat proteins, either the g3p, g8p or g6p [64,69,70]. Similar to other selection technologies, phage display has four criteria

platforms; diversity of genetic information; linking the genotype to the phenotype; enrichment of the selection; and production. Antibody phage display employs the insertion of DNA coding for the variable region alone or with the constant region of antibodies producing functional protein fused with one of the coat proteins of the progeny phage. The displayed antibody fragment is commonly located on the N1 domain of the g3p and rarely on the g6p or g8p.[66,68] The encoded antibody fragment genotype is linked to the phenotype displayed on the phage coat protein.[64,66]. Insertion of recombinant DNA into the phage genome produces only phage with the recombinant phage genome; in contrast the use of a phagemid vector supplying the recombinant DNA which produces both wild type phage and recombinant phage.[66]

Phagemids are hybrids of plasmid and phage vectors, which contain both M13 and *E. coli* origin of replication, the gene 3, 6, or 8, lac promoter, *pelB* leader sequence, ribosome-binding site, and various antibiotic resistant genes with multiple cloning sites. They do not produce complete phage when transfected into *E. coli* because of their lack of the other structural and functional genes. However, co-infection with a helper phage such as M13K07 and VCSM13 phage strains produces both recombinant and wild type phage – termed phage rescue.[66,71] Helper phages usually contain an antibiotic resistance gene to aid in selection of progeny phage that contain both the helper phage genome and the phagemid vector.[66] The progeny phage contains either the g3p-fusion antibody fragment or the helper g3p with a ratio of 1:9 to 1:1000.[64] To increase the ratio of fusion product, helper phage has been molecularly modified. The helper phage M13K07 has a defective origin, and packaging of the helper g3p is lower than the phagemid supplied g3p. Each of the five copies of g3p has the potential to display the fusion protein; however, only one fusion protein is displayed on average. Other systems of phage rescue to combat the problem of wild type over recombinant phage progeny have been developed, such as deletion, mutation, or the introduction of a stop codon or protease site in the helper phage g3. These mutated helper phages (Hyperphage, Ex-phage, and Pharberge) impart the attribute of g3 deficiency production in the expression vector.[72] The most successful system is the one employing two amber stop codon in the g3.[66,72] The use of the g8p as the display protein greatly increases the amount of antibody fragments displayed. However size of the polypeptides is a limiting factor, which makes the use of the g3p preferred to g6p or g8p [71]. Modifying the phage genome to contain the wild type gene 3 and the antibody fragment-gene 3 hybrid eliminates the need for helper phage or phagemid [73]. However, the efficiency of library construction and bacterial transfection is accomplished more easily with phagemid than phage. Filamentous phages are efficient at deleting extraneous DNA whereas the phagemid are more stable.[66] Another advantage to the phagemid system is the ability to produce soluble antibody at higher concentrations by incorporation of an amber stop codon. There are three stop

codons which will terminate the ribosome from translating mRNA – amber (UAG), ochre (UAA), and opal (UGA). Incorporation of the amber stop codon before the g3p will produce the antibody fragment as a soluble protein and prevent it from being displayed as a fusion protein.[71]

The B lymphocyte is unique is its ability to specifically bind antigen by a surface protein complex; the B cell receptor (BCR), which is composed of the membrane-bound IgM or IgD along with CD79a and CD79b. An individual has an immense number of B cells, each with different antigen specificity; the collective population is termed the B cell repertoire. The specific binding of antigen to the BCR activates the cell to proliferate and differentiate into plasma or memory cells, resulting in a clonal expansion of this specific B cell. The plasma cell is an end stage B cell antibody producing factory. The entire antibody it produces has the same antigen specificity as the bound Ig of the originally activated B cell.[74] Resting B cells produce approximately 100 copies of immunoglobulin mRNA per cell; in comparison to the plasma and hybridoma which make over 30,000 copies per cell. Immune selection through mutation enriches those B cells with higher affinity to the antigen. The antibody phage display technology mimics the antibody production of mammalian B cells.[71]

Antibodies are composed of a pair of identical heavy and light polypeptides chains held by non-covalent bonds and disulfide bridges. Genes encoding for the heavy chain are variable (V_H), diversity (D_H), joining (J_H) and constant (C_H). Genes encoding for the light chain are variable (V_L), joining (J_L), and constant (C_L). C genes are conserved within a species; whereas, the V genes are antigen dependent. The variable regions contain three hypervariable regions flanked by the more conserved framework regions. It is these hypervariable regions or complementarity-determining regions (CDRs) that make an antibody specific to a certain epitope. Structural diversity of an antibody's binding site is attributed to the heavy chain.[71] The first two CDRs are encoded by the V segment and the third CDR is encoded by the V-D-J or the V-J segment of the heavy and light chain respectively. Most species produces a great variety of unique antigen-binding sites by several mechanisms such as recombination among the different encoded genes, imprecise joining, deletions or insertions of random nucleotides, or conformational changes of the antigen binding site. The preimmune repertoire of pigs has a limited diversity, possessing two D_H genes, seven V_H genes, and a single J_H gene; therefore the CDR3 determines the BCR diversity. The V_H genes of pigs belong to the V_H3 family where the framework regions are identical.[53,75] Pigs undergo class switch recombination without somatic hypermutation through gestation in the absence of environmental antigen and therefore they have a germline (preimmune) repertoire which consists of IgG, IgA and IgM producing B cells.[76,77] Antibodies can be digested or produced by recombination into discrete protein domains or fragments. Cloning the genes that code for the V_L and V_H produces a variable fragment (Fv) and the V_H-C_H with V_L-C_L

produces the disulfide bonded fragment antibody (Fab). The linked V_H-V_L fragment is termed single chain variable fragment (scFv). A series of amino acids form a linker between the V_L and V_H segments to allow for conformational association of the two peptides and the formation of competent antigen binding sites in a single protein.[64,66] The advantages of scFv over Fabs are several. The DNA insert coding for the Fab are larger which increases the probability of construction problems, increased instability and degradation, and lower yields of soluble proteins have all been encountered.[66] Different forms such as dimers of scFv or diabody are produce by inserting a short linker of approximately 5 amino acid residues between V_H and V_L domains which prevents alignment of a single scFv. Diabodys contain two functional antibody binding sites and increases the avidity of the antibody to the antigen. Decreasing the size of the linker will form tribodys (scFv trimer)[66,67]. Manipulation of the amino acids within the antigen binding site by site-directed mutagenesis to favor parental sequences or those appear more frequently in CDR regions of antibody to select for antibodies of higher affinity. Production of ScFv is suited for clinical therapeutic agent applications, because of their lower retention in non-target tissue, swift clearance from peripheral blood circulation, and increased tumor invasion.[78]

Antibody Phage Display Library Construction

A phage display antibody library is a powerful tool for isolation of recombinant antibodies with a unique specificity. Phage displayed recombinant antibodies have been used for immunotherapy, drug discoveries, functional studies such as protein-protein interactions and development of clinical assays.

There are three types of antibody phage libraries, naïve, immune and synthetic. The naïve and immune libraries rely upon species-specific B lymphocytes expressing antibody transcripts; whereas, the synthetic library uses molecular techniques and randomization of transcripts. The naïve library is constructed from non-immunized animals. The variable genes of IgM mRNAs of B lymphocytes isolated from peripheral blood, bone marrow, tonsil and spleen. The B cells of each area of tissue or peripheral blood is at a different stage of maturation which affects the diversity of the variable genes amplified. The most naïve population of B cells resides in the bone marrow; the most mutated the tonsils and the most immune or memory cells being within the secondary lymph nodes. Although peripheral blood contains naïve and immune B cells, the latter population expresses higher concentrations of mRNA, therefore more mutated variable regions.[66] Naïve libraries increase the likelihood of antibodies with lower immunogenic properties such as self-antigens; however, lower affinity and diversity of antibodies have been observed. The immune library is constructed from immunized animals and the variable genes from IgG mRNAs. This type of library will produce

14

antibodies specific to the antigen and possible affinity maturation will have occurred in the animal's immune system.[67] Undoubtedly the antibody repertoire of an immune library will have a greater affinity to the antigen target than the repertoire of a naïve library.[66] The immune library takes more time to construct due to immunization of the animal. Also transcripts are skewed towards the immunized antigen; therefore isolation of antibodies specific to other antigens may not be successful. A synthetic library is made by randomization of the heavy chain or light chain segments of the mRNA transcripts. Direct mutagenesis kits and PCR techniques are utilized to change the CDRs of the different chains which increases the diversity and variability of the different antibody fragments.[67,69,71] Initial constructions of synthetic libraries were plagued with low affinity antibodies; however, selections of functional variable regions have remedied this problem. The synthetic library has the advantage of a selection of variable genes that are optimally expressed in *E. coli*.[66]

The mRNA transcripts are reverse transcribed to produce the complimentary DNA strand. Polymerase Chain Reaction (PCR) is performed using species specific primers that recognize canonical antibody sequences. The database VBASE (www.mrc-cpe.cam.ac.uk/PRIMERS.php?menu=901) and IMGT (http://imgt.cines.fr/) contains several mammalian variable, diversity and joining gene sequences, allowing for easy and accurate primer construction.[66,79] In theory the entire antibody repertoire of a species can be amplified in this manner. The products can then be assembled into a single gene. The assembled linked V_H and V_L gene fragments and V_H-C_H with the V_L-C_L linked by DNA containing the ribosome binding site and leader sequence are constructed into a scFv and Fabs, respectively.[66] The nucleotide sequence fragment can then be inserted into a phagemid vector or the genome of the filamentous phage by ligation enzymes. To accomplish insertion of the genes different methods have been established. The first is the amplification of the variable regions of the V_H and V_L with linker attachment by PCR. The V_H-linker fragments to the linker-V_L fragment are hybridized into one fragment with subsequent PCR amplification. Concentrations of the two amplified fragments are important for successful linking. Secondly, a method which clones the amplified variable fragments (V_H and V_L) into two different vectors and then combines the two with restriction enzymes and ligation into separate bacterial expression vectors. The combining step can be changed by combining the two into the same vector. Lastly, the method of cloning a recombination signal such as LoxP into the separate V_H and V_L vectors. The *E. coli* is transformed with the two vectors and the addition of the enzyme, *cre* recombinase causes recombination of the different fragments.[66] The phagemid vector is transfected into a culture of *E. coli* bacterium by either chemical transformation or electroporation. The size of the library is dependent on the efficiency of both ligation of the different scFv and the transformation of the *E. coli.*

15

The library construction using filamentous phage as the vector is dependent on the efficiency of the phage to infect the *E. coli*, the organization of the variable genes, and the strain of the bacterium.[80] The bacteria containing the phagemid are grown to the optimum concentration and helper phage is added to produce complete phages displaying the different scFv on either the g3p, g6p, or g8p coat protein.[71] Phage proteins and antibodies replicate separately and assemble at the inner membrane of the bacteria; since the phage is not lytic, the bacteria secrete the bacteriophage. Each of these phages displays a different antibody on its coat protein and has the potential to bind to a separate distinct receptor or epitope.[49]. The secreted phages constitute the Phage Display Library. Most libraries are limited to approximately 10^8 antibody repertoires due to the efficiency of transformation of the phagemid into the bacterium. However, combinatorial infection of a phagemid containing the heavy chain fragments with a plasmid containing the light chain fragments is transformed into the same bacterium resulting in a chain shuffling of the VH and VL chains. These combinatorial libraries increase the different combinations of chain pairing not seen in the immune system and increasing the diversity of the library.[71] Other methods to increase the diversity is taking advantage of error prone PCR, transfection of a mutator strain of *E. coli*, and mutagenesis.[66,81,82] In vivo recombination produces a repertoire of 10^{10} clones. The quality of the constructed library is tested by the number of clones containing a phagemid with a scFv insert, displaying the scFv, and production of soluble scFv. Screening the clones is accomplished by PCR or dot blot analysis of both phage and soluble scFv. Factors that influence the level of displayed antibody include the antibodies ability to resist aggregation and proteolysis, correct periplasm conformational folding and toxicity to the bacterial cell and whether the bacterial cell are capable of exporting the displayed antibody phage.[66,70] The length of the linker and organization of the V_H-V_L domains plays an important role in the production and folding of the fragment antibody.[80]

Phage Library Screening and Isolation of Specific Antibodies

Phage display libraries are screened to select for antibodies to a specific target antigen. This selection process is termed panning and consists of several steps to obtain a specific avid antibody to the antigen of choice. The first step is to screen the phage display library for specific binding to the target antigen. There are several ways to perform this step, such as immobilizing the antigen onto the surface of a plastic surface (beads or plates) or affinity column, capture methods with avidin coated beads with the antigen in solution, selection on cells, and filter paper from 2D gels or high density Western Blots. High throughput methods have also been developed, which utilizes the multiplex bead assay technology, and decreases the amount of target antigen needed for screening. This method enables the screening of

multiple target antigens at one time without the need for precipitation of produced phage.[83,84] Paramount to selecting a specific antibody to the target antigen is the purity of the target.[66] Often the target antigen or protein is not available for screening and recombinant peptides are produced to screen the libraries. The next step is to remove unbound and low affinity antibody fragments with repeated washing with a low ionic buffer leaving only the antigen specific fragments. The addition of BSA, 2% milk powder, gelatin or casein reduces nonspecific binding by blocking areas of the surface. A phage display library with a diversity of 10^{8-10} theoretically has an efficiency of 10%, meaning a low number of copies of each displayed antibody.[66] The bound specific antibody is eluted from the antigen. An acidic or basic solution, enzymatic cleavage, or excess antigen releases the antibody displaying phage from the antigen. Panning or first round affinity selection is the most important step to obtaining a target specific antibody. The recovered antibody displayed phage is then infected into another culture of *E. coli* and the process begins again. Subsequent rounds of selection with a decreased concentration of target antigen increases the immune enrichment of the antibody fragment [66,71]   Panning selection is commonly performed 3-7 times to isolate and amplify the antibody of choice and to minimize non-specific antibodies.

Soluble scFv are produced by infecting a nonsuppressor strain of *E.coli*. The phagemids are engineered to contain an amber stop codon between the scFv genes and the 3 protein gene.[69,71] *E. coli* that contains the *supE* gene does not recognize this as a stop codon and will continue to translate the protein creating a fusion protein. However, nonsuppressor strains such as HB2151 or Top10F' recognizes the AUG as a stop codon and stops the translation producing a separate scFv from the phage particles. Phagemids contain a LacZ promoter and expression of the soluble antibody fragment is driven by the addition of IPTG.[66] The addition of a polyhistidine or myc tag upstream from the scFv gene produces a fusion product with the scFv and allows for purification of soluble scFv fragments by metal affinity chromatography. These soluble antibodies can be found in the supernatant, bacteria periplasm or cytoplasm of the bacterial cell. Therapeutic antibodies are produced in this fashion.

Applications and Diagnostics

The applications of phage display are many, from discoveries of novel protein interactions to therapeutic treatment with recombinant antibodies or enzymes. Recombinant antibodies are comparable to monoclonal antibodies when used for traditional applications such as immunofluorescent assays, western blotting and immunohistochemistry. Phage produced random peptides libraries are used to discover binding sites of wide range of different targets, map epitope binding sites, and used to identify enzyme substrates interactions. The most successful use of phage display has been the isolation and

production of specific antibodies to target antigens.[66,71,85] Also because the bacteriophage is immunogenic, potentially they could be used as vaccine candidates when displaying the neutralizing peptide of an infectious disease such as PRRSV. The immune response to the M13 phage was shown to be T-cell dependent and bypasses the need for an adjuvant.[86,87] Random peptide libraries are used to identify antibodies in patient's serum after infection with an unknown agent. Negative sera were screened to isolate only those peptides that were specific to the infectious agent. Researchers developed diagnostic assays to identify the infectious agent in other suspected patients without knowledge or isolation of the disease causing agent.[88]

Recombinant antibodies produce by phage display is advantageous over the historical hybridoma production of monoclonal antibodies. Hybridoma technology starts with the immunization of mice, relying on an immune response to the antigen, recovery of cells from the spleen of the immunized mouse, screening the cells for production of the specific antibody to the antigen and once discovered fusion to the myeloma cell line for the continued production of the antibody. Monoclonal antibodies have been produced this way since the discovery of the technique by Dr. Roger Milstein. The technique is labor intensive and involves the maintenance and care of mice, cell culture and is time consuming. The technique cannot be used to produce antibodies to non-immunogenic or toxic antigens, because the mice being immunized do not mount a response or succumb to the toxin before an immune response is elicited. Antibodies to self-antigens such as the major histocompatibility complex (MHC) or blood groups, which were difficult at best with hybridoma techniques, have successfully been produced by phage display.[89] Phage or phagemid genes can be manipulated by molecular techniques and does not involve the care and use of an animal. Monoclonal antibodies are mouse produced antibodies and have the potential to illicit an immune response if used for immune therapy; whereas the recombinant antibodies produced by phage display are species specific and less likely to stimulate a response.

Novel enzyme inhibitors and antagonist were discovered by the use of phage displayed proteins. Functional enzymes have successfully been displayed on the surface of the M13 phage, such as Trypsin and alkaline phosphatase and when coupled to a scFv, the potential to have one step detection diagnostic exists.[90] A fusion of scFv with green fluorescent protein (GFP) has been developed for use in flow cytometry cell staining.[91]

Phage displayed antibodies have been used in anti-tumor therapy. The small size of the scFv antibody fragment has better tumor penetration with more rapid clearance than full size antibodies. The fusion of scFv with radio-isotopes or toxins delivers the drug to the site of the tumor growth without affecting the surrounding cells or tissue. Phage derived Fab antibodies are used as prophylaxis and/or treatment of viral infections. Bispecific antibodies (BsAb) contain two different binding sites to

different epitopes on an antigen or target protein. Diabody formation of two different V_H and V_L chain scFv to the same antigen but different epitopes show higher avidity than the single scFv antibody. Phage display offers a mechanism to alter the genetic code of the phagemid expression vector, in such a way that a non-canonical amino acid would be utilized within the V_H CDR3 and increase its effectiveness to bind antigen.[92]

The rabies virus is a fatal disease for humans and domestic animals. Human post exposure is managed by vaccination in non-immune or the administration of serum derived polyclonal antibodies in previous vaccinates. In the past, a major disadvantage of the post exposure prophylaxis is the availability, immune reactions, variations in production and contamination with other blood borne diseases. Human antibody phage display libraries have been screened and fragments that were specific to the rabies virus were identified. Several were found to neutralize the rabies virus's ability to infect cells *in vitro*. Binding competition studies comparing the phage derived antibody fragments to that of human vaccinate derived antibodies demonstrated that they bound to two different binding domains of the virus. The possibility that selection amplified fragments towards a particular set of epitopes or the immunogenicity of one domain over another may be different between individuals. Production of these phage produced specific antibodies into a therapeutic cocktail could decrease the disadvantages seen with other prophylaxis.[93]

Figures and Tables

Figure 1.1: Structure and Replication Schematic of PRRSV Genome
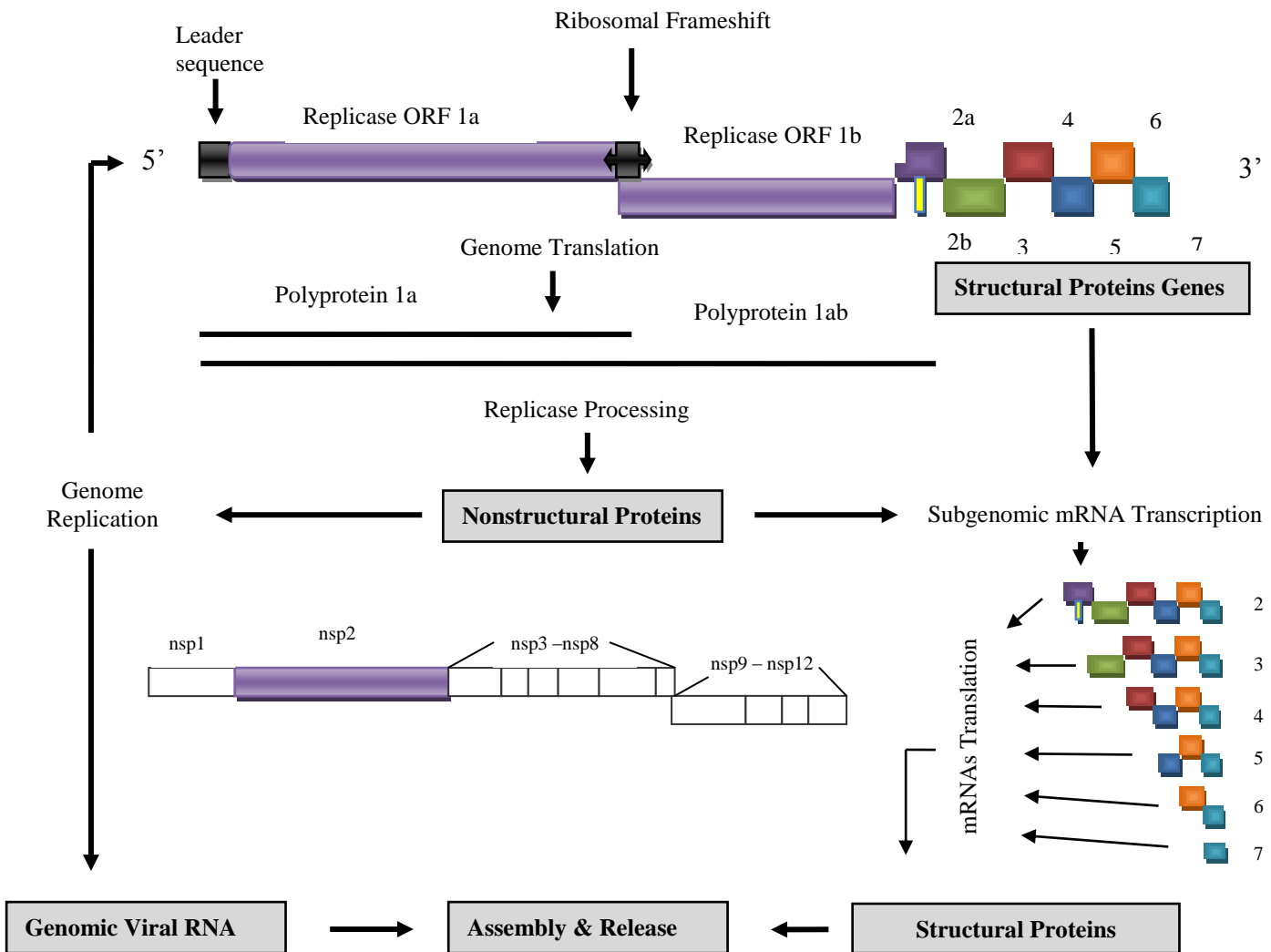
Figure 1.1: Schematic of PRRSV genome as previously reported by *Music et al.* with modifications.[11] Upon entry of the virus genome into the cytoplasm of the cell, immediate translation of ORF1a and through a ribosomal frameshift ORF1b occurs. Polyproteins 1a and 1ab are produced, processed by *cis* and *trans* cleavage and function in the replication and transcription of subgenomic RNAs. Structural proteins are produced by translation of subgenomic mRNAs.

Figure 1.2: PRRSV nsp2

Figure 1.2: Schematic of the PRRSV nsp2. *Denotes reported epitope sites in SD01-08 by *Chen et al., 2010*.[1]

Figure 1.3: pHEN2 phagemid vector

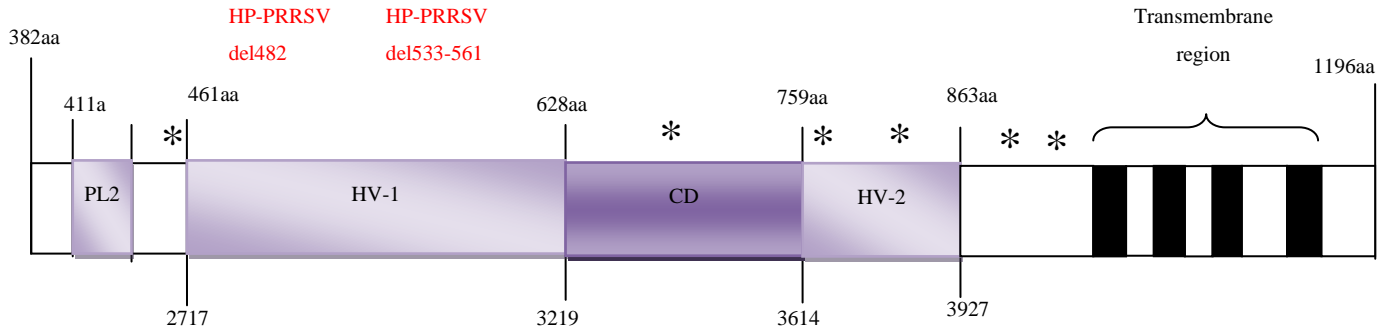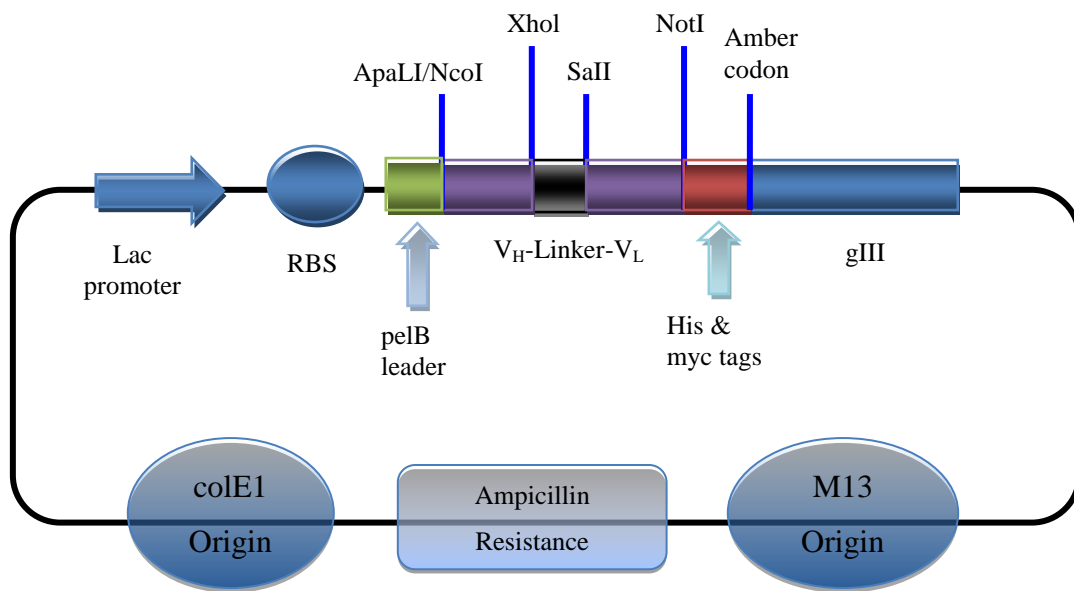Figure 1.3: Diagram representing the pHEN phagemid vector containing pig VH-VL inserts.

Chapter 2 - Characterization of Single Chain Variable Antibody Fragments (ScFv) specific to the Porcine Reproductive and Respiratory Syndrome Virus Nonstructural 2 (nsp2) Fragment Protein

Abstract

The Porcine Reproductive and Respiratory Syndrome Virus has plagued pork producers since the late 1980s. The quest to produce a vaccine that would enable the diagnostic identification of a pig infected with wild type virus from a vaccinated pig is the focus. Sequence analysis demonstrates that the nsp2 protein possesses the ability to remain functional with mutation, insertion and deletion of large areas within the protein. Several studies have focused on this unique ability and produced vaccine strains containing reporter proteins such as GFP within this non-critical area of the nsp2 protein. In this study, porcine displayed scFv fragments which were constructed by the laboratory of Dr. Robert Aitken from pig splenic cDNA was screened for specificity to a recombinant fragment protein of the PRRSV nonstructural protein 2. Nine single chain variable fragments (scFv) were identified to specifically bind. The phagemid DNA containing the nine scFv insert were purified and sent to Dr. Carol Wyatt for further characterization. The nine scFv antibody fragments were recovered by phage display techniques and panned for a total of four times against the recombinant PRRSV nsp2 fragment protein. Initial ELISAs were performed with the recombinant protein only, after the third round of panning all scFv were assessed for binding to recombinant ubiquitin from the pHUE vector and a commercial source of human ubiquitin for specificity by ELISA and Western Blot.   All nine scFv were found to specifically bind to the ubiquitin tag contained in the recombinant PRRSV nsp2 fragment protein. Immunoflourescent assays were performed on PRRSV infected MARC-145 cells to discern whether binding of the nine scFv could possibly recognized the virus produced nsp2, none were found to bind; supporting results attained by the ELISAs.

Introduction

Porcine Reproduction and Respiratory Syndrome Virus emerged in the late 80s with large scale infection amongst pig producing areas of the US, Europe and Asia. This once "mystery disease" caused losses in breeding programs due to aborted fetuses and post weaning pneumonia with a lower performance in surviving piglets.[2-5] The PRRSV is an enveloped, single positive stranded RNA virus grouped with Equine Arteritis Virus (EAV), Lactate Dehydrogenase Elevating Virus (LDV), Porcine

Reproductive and Respiratory Syndrome Virus (PRRSV) and Simian Hemorrhagic Fever Virus SHFV into the family *Arteriviridae*, Order *Nidovirales*.[16] These four viruses share similar molecular and biological properties. They exhibit a wide range of disease symptoms, from relatively asymptomatic (LDV) to death (SHFV, PRRSV).

Possessing a polycistronic genome, PRRSV is approximately 15.2 kilobases (kb) with a polyadenylated 3'end and a type I 5'cap structure, which can be replicated, transcribed , or translated immediately on entry into host cells. Thirteen nonstructural proteins are encoded in two open reading frames (ORFs 1a and 1ab) occupying three quarters of the genome. Structural genes are encoded in the remaining one third of the genome and are translated from nested subgenomic mRNA. A characteristic shared by all nidoviruses.[4,11] There are three areas of the PRRSV genome that show the most variability – the structural genes encoding GP_5 and GP_3 along with the nonstructural gene encoding the nsp2 protein.[30] The nsp2 protein containing approximately 1100 amino acids is the largest of the nsps and has the most genetic differences between the two PRRSV genotypes (NA and EU strains).[39,40] Within the genome of the nsp2 is three domains; an N-terminal cysteine proteinase domain (PL2), a middle region, and a hydrophobic transmembrane region. Sequence analysis of field strain PRRSV has shown natural mutations, insertions and deletions in the middle domain, while the N and C terminal domains are conserved.[40] Vaccine development has focused on the unique ability to withstand drastic variability in this area of the nsp2. Marker vaccines have been produced by inserting a GFP fluorescent tag within a nonessential area with minimal loss of viral fitness.[30,41,42] There are two areas of hypervariability (HV-1 and HV-2) flanked by conserved sequences of amino acids within the middle domain. Reverse genetics studies have shown that the PL2 and transmembrane domains are essential for viral growth but that deletions as great as 309 aa in the middle domain affected only growth kinetics.[40] Interestingly, the emergence of a highly pathogenic PRRSV strain in China and parts of Asia, with a discontinuous deletion of 30aa in the HV-1 region of the nsp2 domain , has suggested that the middle domain may play a part in the virulence of the strain.

In PRRSV infection of isolator piglets showed a dysregulation of the immune system response. A large number of the antibodies produced had hydrophobic CDR3s, suggesting an expanse of immature B cells. Previous studies employing phage technology have mapped several linear B cell epitopes to the nsp2 protein. Screening infected pig sera against peptides to these predicted epitope determined that antibodies recognizing the 18 sites were produced.[44] Antibodies to the nsp2 were produced days after infection.[45] In the current study, recovered phagemid DNA containing scFvs were produced and screened for antibody specificity to a recombinant PRRSV nsp2 fragment protein. The recombinant PRRSV nsp2 fragment protein was produced by vector pUb-nsp2 (628-759), provided by Dr. Robert

(Bob) R. Rowland, corresponding to nucleotides 3219-3614 of the PRRSV strain P129, expressing a 5x His-ubiquitin nsp2 fragment fusion protein in the chemically competent *E. coli* strain BL21(DE3).[48] Spleens were recovered from pigs obtained from a slaughterhouse amplified and cloned into the pHEN2 expression vector.(Figure 1.3) Initial screening was performed by visiting scientist Dr. Carol Wyatt, Kansas State University College of Veterinary Medicine. Nine scFv clones were identified – A8, A9, A11, B1, B9, D2, F9, F10, and G3. The purified phagemid DNA containing the nine scFv inserts were recovered by reported phage display techniques. Selection against an expressed recombinant fragment of the PRRSV nsp2 was performed for four rounds of panning. After the third round, ELISAs were performed to assess whether the scFv recognized recombinant pHUE expressed ubiquitin and recombinant human ubiquitin. Western Blots were performed to characterize the scFv. To determine binding was occurring at the junction of the ubiquitin and nsp2 fragment synthetic Peptides were produced. These peptides corresponded to amino acid sequences of the nsp2 fragment were produced to determine if the binding site could be discovered. Peptide 1(LHLVLRGGRPKYSAQAII) matches the sequence of amino acids linking the ubiquitin protein to the nsp2 fragment protein; Peptide 2 (LHLVLRGGDLGGPCSGHL) is made up of same amino acids of the C'terminal end of the ubiquitin protein, skipping an 11 amino acid stretch, to the next 10 amino acids of the nsp2 fragment; and Peptide 3(SVSAESDLTIGSVATEDIPRILGKIENTGE) corresponds to the C-terminal end of the nsp2 protein and contains a reported linear B cell epitope reported by *de Lima et al.* (PRILGKIEN). Western Blot analysis showed high background with secondary antibodies and could not detect unquestionable binding of scFv to the different peptides; binding to recombinant PRRSV nsp2 fragment protein was not evident. ELISAs were performed by coating 96 well plates with the nsp2 fragment, expressed pHUE ubiquitin, human ubiquitin and recombinant GFP-ubiquitin fusion protein. Results revealed the nine scFv initially thought to be specific to the nsp2 fragment were in fact specific to ubiquitin from both the pHUE expressed and human ubiquitin.

Materials and Methods

Cells

Escherichia coli bacterial strain TGI: K12 [A (lac-pro), supE, thi, hsdD5/F'traD36, proA+B+, lacIq, lacZAM15] was received on dry ice from manufacturer and directly placed at -80^0C until used in experimentation. The *E. coli* BL21 Competent cells [F– ompT gal dcm lon hsdSB(rB- mB-) gal [malB+]K-12(λS)] containing the expression vector pUb-nsp2 was stored at -80^0C, cells were immediately thawed on ice prior to inoculation into Luria Broth for protein fragment expression.

MARC-145 cells, a descendant of the MA-104 cell line (African Green Monkey kidney cells) were grown and sustained in Eagles' MEM with 7% FBS, supplemented with 0.01% penicillin-streptomycin, which were used to assess the ability of the scFv to bind to nsp2 protein *in vitro*. The PRRS infectious clone construct pCMV-S-P129-1bMCS2 passage 5 which contain the modification of the reporter insert changed from GFP to RFP.[94]

Culture Media

Luria-Bertani Broth (LB) and or Luria-Bertani Agar (LA) is prepared by mixing 10g of Bacto Tryptone (Becton Dickinson, cat # 211705), 5 g of Bacto Yeast Extract (Becton Dickinson, cat#212750) and 10 g of NaCl (Sigma, S3014) into 1 liter of doubled distilled filtered H_2O, autoclaved and aliquoted into 500mL sterile bottles. Luria Agar is prepared the same as LB with the addition of 15 g of Agar, Grade A (BBL, cat# 212304), autoclaved and poured into culture plates.[78]

Super Optimal Broth (SOC) prepared for transformation of phagemid vectors after electroporation by mixing 2g of Bacto Tryptone, 0.5 g of Bacto Yeast Extract, 1mL of 1M NaCl, .25mL of 1M KCL (MCB Reagents, PX1405), and 1mL of 2M D+-Glucose Monohydrate (Fluka Biochemika, cat#49159) with 1 liter of double distilled filtered H_2O, autoclaved and aliquoted into 500 mL bottles.

2xTY Media was prepared by mixing 16 g of Bacto-Tryptone, 10 g of Bacto Yeast Extract, 5g of NaCl with 1 liter of doubled distilled filtered H_2O, which was aliquoted into 500 mL bottles and autoclaved. 2xTY agar plates were prepared as above with the addition of 15 g of Agar, Grade A, autoclaved with the addition of 100 μg/mL of Ampicillin and 1% or 0.1% Glucose poured into culture plates.

TYE agar plates is prepared by adding 15 g of Agar, Grade A, 8 g NaCl, 10 g of Bacto-Tryptone, and 5 g of Bacto-Yeast extract into 1 liter of doubled distilled filtered H_2O, autoclaved and poured into culture plates.

Expression of Recombinant Proteins

The expression vectors pUb-nsp2 (628-759), pHUE-ubiquitin and pUb-GFP were provided by Dr. Robert (Bob) R. Rowland. The pUb-nsp2 is a pHUE vector backbone with the cDNA fragment corresponding to nucleotides 3219-3614 of the PRRSV strain P129 flanked by SacII and BamHI restriction endonuclease cleavage sites. The pHUE-ubiquitin and the pUb-GFP have the same pHUE

backbone with cDNA inserts coding for the ubiquitin and GFP proteins. These expression vectors all contain a 5x His fusion protein in the chemically competent *E. coli* strain BL21 (DE3) (Invitrogen, cat# C6000-03).[48]

Protein Fragment Expression

Expression of recombinant PRRSV nsp2 fragment protein, pHUE-ubiquitin, and pUb-GFP was accomplished by following protocol outlined by *Catanzariti et al.* [95] *E. coli* strain BL21 was inoculated at 1μL/10 mL of sterile LB media containing 100 µg/mL of ampicillin antibiotic and grown shaking (250rpms) at 37^0C overnight. The resulting culture was poured into 200 mL of sterile LB media containing 100 µg/mL of ampicillin and grown to OD600 of 0.4-0.6, approximately 2-4 hours. 2 mL of 0.1M isopropyl thiogalactoside - IPTG (Sigma, cat# I5502) was added to the 200 mL culture and left to incubate an additional 2-4 hours. The induced culture was spun at 4000 x g for 10 minutes in a Sorval SS-34 rotor placed into the Sorval RC 28S ultracentrifuge (Beckman) to concentrate the bacteria. Bacterial cellular disruption and clarification was achieved by the addition of LEW buffer, 1 mg/mL of lysozyme, and 6-10 pulses of sonication. The produced suspension was centrifuged at 1300 rpms as mentioned above resulting in a cellular debris button and the released protein lysate. At each step of growth, an aliquot was taken to assess protein production by SDS-PAGE analysis. (Figure 2.1)

Purification of Expressed Recombinant Proteins

Purification of expressed recombinant nsp2 fragment protein, pHUE-ubiquitin, and pUb-GFP was accomplished by following protocols outlined by PrepEase® Histidine-Tagged Protein Purification Kit and *Catanzariti et al.*[95-97] Briefly, a PrepEase nickel (Ni-TED) column was equilibrated by filling with 1x LEW buffer draining by gravity flow into a labeled polystyrene tube. The protein lysate was added to the column. Two wash steps were performed with 1x LEW buffer. To elute the attached nsp2 fragment protein, the column was filled with 1x Elution buffer. The eluate was collected and labeled for SDS-PAGE analysis.

Phage display ScFv selection and Panning to nsp2 fragment Protein

Initial screening of a porcine phage display library against recombinant nsp2 expressed protein was performed by Dr. Carol Wyatt at the University of Glasgow, UK while visiting the laboratory of professor Robert Aitken. The porcine phage display library was cloned into the pHEN2 phagemid vector which contains an ampicillin resistant gene, HIS myc tag, and the V_H and V_L domains linked by two copies of a glycine-serine linker. Several enzyme restriction sites are located within the phagemid vector, Sfil/NcoI between the body of the vector and the V_H domain, XhoI/SaII between the V_H and

linker sequences, and NotI between V_L domain and the body of the vector. Nine different scFv were identified and amplified by two to three rounds of panning. The phagemid vectors for each scFv were sent frozen in phosphate buffered saline 15% glycerol to Dr. Wyatt's laboratory at Kansas State University and stored at -20^0C.

E. coli Transformation

Selection of PRRSV nsp2 protein specific scFv fragments by phage display were performed by following the Tomlinson I+J library procedures with modifications.[98] PRRSV nsp2 scFv phagemid vectors were thawed on ice and 3 µL of each were transformed into 40 µL *E. coli* bacterial strain TG1 (Stratagene, cat #200123) by one pulse electroporation at 1700 V/ 25Ω in sterile electroporation 0.1 cm gap cuvettes (Fisherbrand, cat# FB101). Immediately 960 µL of sterile 37^0C SOC media was added to the cellular-phagemid mixture, which was transferred to a 14 mL polypropylene round bottom sterile tube (Becton Dickinson cat#352059) and placed within a 37^0C incubator shaking at 250 rpm for one hour. After incubation, 10 and 100 µL of transformed bacteria were plated onto TYE agar plates containing 1% glucose (Invitrogen, 15023-021) and 100 µg of ampicillin sodium salt antibiotic (Sigma, cat#AO166), streaked with a sterile spreader and placed at 37^0C for overnight incubation. Individual colonies were picked from overnight culture and inoculated into 3 mL of sterile 37^0C 2xTY agar containing 1% glucose and 100 µg of ampicillin and incubated at 37^0C for 16-18 hours, after which, cultures were centrifuged at 2800 rpms for 15 minutes to pellet *E. coli* cells containing the scFv phagemid vectors, discarding the supernatant.

Purification of scFv phagemid DNA

Pelleted *E. coli* cultures were purified by PureYield Plasmid Miniprep System (Promega cat# A2393). 600 µL of TE buffer was added to pelleted cultured cells and lysed with 100 µL of Cell Lysis Buffer in a 1.5 mL microcentrifuge tubes. The tubes were inverted 6 times to mix and complete cellular lysis was observed. After a maximum of two minutes, 350 µl of cold (4–8°C) Neutralization Solution was added and mixed thoroughly by inverting. The tubes were centrifuge at 13,000 rpms in a microcentrifuge for 3 minutes. Supernatants were transferred to a PureYield™ minicolumn on a Luer-Lok® adapter of a VacMan® Laboratory Vacuum manifold without disturbing the cellular debris pellets. Vacuum was applied to pull lysate through the columns. 200µl of Endotoxin Removal Wash was added to the minicolumns. To wash, 400µl of Column Wash Solution was added to the minicolumns. The minicolumns were removed from the VacMan® Laboratory Vacuum manifold, placed into 2 mL collection tubes, and centrifuge at 13,000 rpms in a microcentrifuge (Accuspin, Fisher

Scientific) for 1 minute. The minicolumns were transferred to clean 1.5mL microcentrifuge tubes. To elute the purified phagemid vectors, 30µl of Elution Buffer or nuclease-free water was added directly to the minicolumns matrix and let stand for 1 to 3 minutes at room temperature. A final centrifugation at 13,000 rpms for 15 seconds eluted the scFv phagemid DNAs. The concentrations of the eluted DNAs were assessed by the Nanodrop 8000 UV spectrophotometer (Thermo Scientific).

Restriction Endonuclease Cleavage of V_H-linker-V_L Insert

Phagemid vector DNA was assessed by restriction enzymes, NotI (New England Biolabs, cat# R0189S) and NcoI (New England Biolabs, cat# R0193T) , for V_H-linker-V_L insert. Restriction endonuclease (RE) cleavage was performed for identification of bacterial clones containing a phagemid vector after ampicillin antibiotic selection following manufacturers suggested procedure. The NotI restriction endonuclease ($GC^vGGCCGC$) was added to each DNA preparation at 1µL/1µg of DNA with 1µL of 10x buffer 3, 1 µL of bovine serum albumin and 9 µL of RNase free water into a 1.5 microcentrifuge tube. Microcentrifuge tubes were placed in a 37^0C heating block (Accublock Digital Drybath, Labnet International, Inc.) for two hours. At which time, NcoI restriction endonuclease (C^vCATGG) was added to each DNA preparation at 1µL/1µg of DNA in each microcentrifuge tube. Tubes were incubated in the 37^0C heating block for an additional two hours. Tubes were removed and assessed by agarose gel electrophoresis using a 1.5% agarose gel with 2 µg/mL of Ethidium Bromide. Those clones containing the appropriate size band of the V_H-linker-V_L insert were selected for amplification and production of scFv. (Figure 2.2)

Polymerase Chain Reaction (PCR) of purified phagemid DNA

Phagemid vector DNA was amplified by "Hot Start" PCR to identify selected clones with V_H-linker-V_L insert. 20pmol/µL of sense primer labeled LMB3 (5'- CAGGAAACAGCTATGAC - 3') with antisense primer gIII (5'-CCCTCATAGTTAGCGTAACG - 3') was added to a PCR reaction tube containing approximately 10 ng of purified DNA, 5 µL of 10X PCR buffer, 2 µL of 25mM Mg^{2+}, 2 µL of 10 mM dNTPs and 34.5 µL of RNase free H_20 and placed into a 96^0C Techne TC-3000 thermocycler. Samples were brought to 96^0C for 5 minutes and then 0.2 µL of 5U/µL Platinum Taq DNA polymerase was added to each reaction tube. A total of 30 cycles of 30 seconds at 94^0C to denature, 30 seconds at 55^0C to anneal primers, followed by 2 minutes and 30 seconds of elongation at 72^0C. Samples were then assessed for a product of approximately 1.0kb by 1.5% agarose gel electrophoresis. (Figure 2.4)

Production of scFv displayed phage

The clones containing the appropriate size of a DNA scFv insert were inoculated into 200 mL of pre-warmed 2xTY media containing 1% glucose and 100 µg/mL of ampicillin and grown in a shaking (250 rpm) dry incubator at 37^0C until the OD600 was 0.4, approximately 1-2 hours. 50 mL of bacterial clone cultures were removed, leaving the remaining 150 mL of each culture in the incubator for an additional two hours. After the two hour incubation the remaining stock cultures were spun at 10,800 x g, liquid decanted and the bacteria suspended in 10 mL of 2xTY with 15% glycerol, aliquoted, and stored at -70^0C. The removed 50 mLs of culture were inoculated with 10 µL of $\geq$ 1 x 10^{11} pfu/mL M13K07 helper bacteriophage (Invitrogen, cat#18311-019) and placed into a 37^0C water bath for 30 minutes to allow helper phage infection of selected clones. The bacterial cultures were removed and centrifuged at 3200 rpms for 15 minutes and all liquid was decanted from the bacterial pellets, which were suspended in 100 mL of sterile pre-warmed 2xTY containing 0.1% glucose with 100 µg/mL of ampicillin and 50 µg/mL of kanamycin and placed into a shaking (250 rpms) incubator at 30^0C overnight. The next day, cultures were removed and spun at 3600 rpms for 30 minutes to pellet bacteria and cellular debris, which was discarded. To 80 mL of phage containing culture supernatants 20 mL of a 20% Polyethelene glycol 6000/2.5 M NaCl solution was added and placed on ice for one hour to precipitate progeny phage. To concentrate the precipitated phage preparations were centrifuged at 3600 rpms at 4^0C. A white pellet was visible and the liquid was decanted. An additional 5 minute centrifugation was performed at 3600 rpms to remove remaining liquid. The addition of 4 mL of PBS 15% glycerol to the phage pellet caused the phage to solubilize, which was then centrifuged at 11,600 rpms in a microcentrifuge to remove any remaining bacterial debris. These progeny phage preparations were titered by making 1 to 100 dilution and subsequent serial dilutions in PBS (1 µL of phage to 10 µL of sterile PBS), with the remaining phage labeled and stored at -70^0C. To each of the 100 µL dilutions of progeny phage 900 µL *E. coli* TG1 strain at an OD600 of 0.4 was added and incubated for 30 minutes in a 37^0C water bath, after which 10 µL of each dilution preparation was spread onto a TYE plate containing 1% glucose and 100 µg/mL of ampicillin. The inoculated TYE plates were placed into a 37^0C incubator and left overnight. The next morning plates were removed and bacterial colonies were counted for each dilution to determine the phage titer. (Figure 2.6)

Selection of scFv displayed phage specific to PRRSV nsp2 protein

Immunotubes (Nunc, cat#444474) were coated at room temperature (21-25^{0}C) with 4 mL of 10 µg/mL of recombinant PRRSV nsp2 fragment protein in PBS on an end over end rotational mixer. Tubes were decanted and washed with PBS 3-5 times by filling tube and immediately decanting. Tubes

were filled with 2% powdered milk in PBS solution to block immunotubes and prevent the binding of non-specific scFv, at room temperature for 2 hours or overnight at 4^0C. Tubes were decanted and washed 3-5 times with PBS. First round selection - progeny phages were panned against the prepared PRRSV nsp2 coated immunotubes by adding 10^{13} concentration of phage in 4 mL of 2% powered milk and incubating for 60 minutes at room temperature on the rotational mixer. Tubes were incubated an additional hour at room temperature without rotational mixing. Immunotubes were then decanted and washed by filling tube and immediately decanting for 20 washes with PBS (TPBS) containing 0.1% Tween 20 (Fisher Scientific, cat# BP337-100). The tubes were blotted with Kimwipes (Kimberly Clark, cat# 06-666-1A) to remove excess TPBS on the last wash. To elute the bound nsp2 fragment specific scFv displayed phage, 500 µL of 1 mg/mL of trypsin (Promega, cat# V5113) in PBS was added and incubated for 10 minutes at room temperature with rotational mixing. The eluted phage was collected and aliquot into 1.5 microcentrifuge tubes and stored at 4^0C. A titration was performed by inoculating a 1.75 mL culture of *E. coli* TG1 strain bacteria at OD600 of 0.4 with 250 µL of eluted phage, incubated for 30 minutes in a 37^0C water bath. To determine titer, the phage infected TG1 bacteria were serial diluted out to 10^8 and plated onto TYE agar plates containing 1% glucose and 100 µg/mL of ampicillin. The remaining TG1 cultures were centrifuged at 11,600 rpms for five minutes, decanted, and suspended in 50 µL of 2xTY media and plated onto a TYE agar plates containing 1% glucose and 100 µg/mL of ampicillin for subsequent rounds of panning. The TYE plates were placed into a 37^0C incubator overnight. Titers of eluted phage were calculated by counting the number of bacterial clones on each of the serial diluted phage TYE overnight plates. (Figure 2.6) Subsequent rounds of selection – the TYE plates prepared for further rounds of selection were covered with 2 mL of 2xTY 15% glycerol and loosened with a sterile spreader and collected into a sterile plastic tube. A 50 µL aliquot of scraped TG1 bacteria was added to 50 mL of pre-warmed 2xTY media containing 1% glucose and 100 µg/mL of ampicillin, incubated shaking (250 rpms) at 37^0C until OD600 is 0.4, approximately 1-2 hours. The remaining scraped bacteria were stored at -70^0C in a 1.5 plastic freezer tube (Nunc, cat#7634). The TG1 bacteria contained the phagemid/scFv insert specific to the recombinant nsp2 protein; 10 mL of the culture was inoculated with 5 x10^{10} of M13K07 helper phage to produce progeny phage with displayed scFv. After 30 minutes incubation in a 37^0C water bath, the cultures were centrifuged 3600 rpms for 15 minutes, suspended in 50mL of pre-warmed 2xTY containing 0.1% glucose with 100 µg/mL of ampicillin and 50 µg/mL of kanamycin, and incubated shaking (250rpms) at 30^0C overnight to produce progeny phage. These progeny phage were then selected on immunotubes, eluted and titered as previously outlined above. Each of the nine selected single chain variable fragments (scFv) was panned for four rounds against the recombinant nsp2 fragment protein. (Table 2.1)

31

Enzyme Linked Immunosorbant Assay (ELISA)

The scFv displaying phage from the nine selections were screened for specific binding to the recombinant PRRSV nsp2 fragment protein, pHUE ubiquitin, a human ubiquitin and the recombinant Green Flourescent Protein(GFP)-ubiquitin fusion protein. A 96 well plate was coated overnight with100 µL of a 20 µg/mL concentration of each of the proteins, decanted and washed the next day with PBS, and blocked with PBS-3% BSA for two hours at room temperature. After an additional three wash step of the plate, 50 µL of the PEG precipitated phage in PBS-3% BSA was added to the wells. Each of the nine scFvs were screened after each panning by this method. The plate was incubated two hours on a shaker at room temperature, decanted and washed five to ten times with PBS containing 0.1% Tween 20 (TBS). A monoclonal antibody to the M13 g8p conjugated to Horseradish peroxidase was diluted 1:5000 in PBS-3% BSA, 100 µL was then added to each well of the plate, incubated at room temperature for one hour, and washed five to ten times with TBS. Identification of bound scFv displayed phage was accomplished by the addition of 100 µL of One StepTMB substrate solution (Thermo Scientific, cat# 34021) to each well and incubated at room temperature for 30 minutes. Color development was stopped by the addition of 100 µL of 1M HCL. The OD was read at 450 nm on an ELISA Plate reader. (Figure 2.8) OD readings above the negative pooled sera were considered positive. Each produced scFv was serial diluted and ELISA was repeated with the nsp2 fragment bound plate. (Figure 2.7)

SDS-PAGE Gel Electrophoresis and Western Blot

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was performed for identification of expressed PRRSV nsp2 fragment protein and identification of phage displayed scFv specific binding to the nsp2 fragment protein.

SDS-PAGE Gel Electrophoresis of Recombinant PRRSV nsp2 Fragment Protein

The expressed recombinant PRRSV nsp2 fragment protein was identified by SDS-PAGE gel electrophoresis. The fragment protein size is estimated to be 25 kDa. All samples collected during the expression of protein to include the flow through, wash and all eluates, were run to identify if the approximate size protein was being produced. Laemmli buffer (Bio-rad, cat#161-0737) was added to 15 µL of sample in a 1:2 dilution and boiled for five minutes. The samples were loaded onto a 12% acrylamide separating gel with a 4% acrylamide stacking gel. A Bio-rad Mini PROTEAN II electrophoresis chamber (Bio-Rad, cat#) was loaded with the acrylamide gel and 1x Tank buffer to cover gel completely for 30-45 minutes at 250 Volts. The gel was removed and washed in distilled

water and microwaved in 30 second increments, with a change of water after two 30 second washes. The gel is stained with Simply Blue ™Safe coomassie G-250 stain (Invitrogen, Cat# LC6060) covering the gel and microwaved 20 seconds and mixed for one hour on a plate rotator, washed with distilled water overnight. Gel was imaged by the UVP GelDoc-It Imaging System with picture development by the Vision Works LS software (Lifesciences) (Figure 2.1)

Western Blot of Phage Displayed Single Chain Variable Fragment (scFv)

SDS-PAGE gel electrophoresis of recombinant PRRSV nsp2 fragment protein is performed as describe above with the exception of protein loading onto a 15% acrylamide gel and the addition of a loading control to each of the proteins of 5 µL of 100 µg of β-actin (Sigma, A3653). The recombinant fragment protein, with and without trypsin digestion, human ubiquitin and three synthetic proteins corresponding to areas of the fragment protein were all loaded onto the gel. (Figure 2.5) All loaded proteins were transferred from the SDS-PAGE gels to Hybond-P PVDF membranes (GE healthcare, RPN1416F) at 100V for one hour in NuPage Transfer Buffer (Invitrogen, NP0006). Membranes were removed and blocked with PBS + 0.1% Tween 20 + 3% powdered milk overnight at 4^0C. Control blots were pooled positive and negative pig sera collected from a previous study of experimentally infected pigs. Sera were diluted 1:400 in 50 mL of PBS + 0.1% Tween 20 + 3% powdered milk (M-TBS). 10 µL of the final panned eluted phage displayed scFv were added to 50 mL of M-TBS at a concentration of 4×10^8 of phage/µL and incubated for one hour at room temperature. The membranes were then washed three times with PBS + 0.1% Tween 20 (TBS) for 10 minutes. For control blots, a 1:5000 dilution of Goat anti-Pig IgG (H+L) Horseradish Peroxidase (HRP) conjugate (Bethyl,) in 50 µL M-TBS was added to the blots and incubated at room temperature for one hour. For scFv blots 1:5000 dilution of anti-M13 HRP monoclonal conjugate (GE Healthcare, cat#27-9421-01) in 50 µL of M-TBS and incubated at room temperature for one hour. All blots were washed four times with TBS for 10 minutes. Supersignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, cat#34095) enhanced chemiluminescent was used to develop positive binding of HRP containing antibody. Visualization of bands was carried out by the Kodak Image Station 4000R. All blots were washed once more and probed for loading control visualization, 1:250 dilution of rabbit anti-actin IgG in 50 µL of M-TBS was added and incubated at room temperature for one hour, followed by 3 ten minute washes with TBS, incubated with 1:10,000 dilution of sheep anti-rabbit IgG (H+L) HRP in 50 µL of M-TBS and incubated at room temperature for one hour, followed by 4 ten minute washes with TBS. Visualization of bands were developed as outlined above. (Figure 2.5)

Immunoflourescent Assay of PRRSV infected MARC-145 cells

MARC-145 cells in media were incubated in a 24 well sterile culture plate at 37^0C at 5% CO_2 for 48 hours. Cells were confluent in each well and infected with pCMV-S-P129-1bMCS2-RFP infectious clone. Once infection had reached approximately 80% of the cells as evidence by emission of red fluorescence, IFAs were performed with all nine of the scFv, non-specific controls and positive binding at room temperature protected from the light. Wells were washed three times with non-sterile PBS, decanted and fixed with 4% paraformaldehyde for 15 minutes. The fixative was removed and cells were gently washed two times with non-sterile PBS. Cells were permeabilized with PBS containing 0.2% saponin for seven minutes and then washed twice with PBS containing 0.002% saponin. Blocking was carried out with PBS containing 2% bovine serum albumin (BSA) for 30 minutes, followed by two washes with PBS – 1%BSA and 0.002% saponin. All antibodies including the scFv were diluted in PBS – 1% BSA with 0.002% saponin. All nine scFv were diluted at 1:2 and nine wells were flooded with 200 µL of each individual scFv dilution, after incubation each well was washed twice with wash solution (PBS – 1% BSA with 0.002% saponin) then mouse anti-M13 (10 µg/mL) was added to each well. An additional two wells containing infected   and on containing non-infected cells were incubated with the mouse anti-M13 antibody to serve as a control for non-specific binding. The SDOW 17purified antibody (South Dakota State University, Brookings, South Dakota) was added at a 1:1000 dilution to an infected well to serve as a positive infection control and to test secondary antibody binding. After 45 minutes, all wells were washed twice with wash solution and 200 µL of 10 µg/mL goat anti-mouse IgG (H+L) FITC was added to wells. The SDOW17 FITC (1:100 dilution) was added to an additional infected well to serve as positive control. A final incubation of 45 minutes and the entire plate was washed twice with wash solution and a final wash with non-sterile PBS. At which time the wells were viewed with a fluorescent microscope with filter sets appropriate for RFP and FITC fluorescent emission. (Figure 2.9)

Bioinformatics analysis

Protein Calculator v3.3 (http://www.scripps.edu/~cdputnam/protcalc.html) determined the Molecular weight of proteins/peptides. Sequence analysis was performed by Vector NTI (Invitrogen) software (www.invitrogen.com). IMGT/V Quest tool at http://www.imgt.org/IMGT_vquest/vquest was accessed for sequence analysis of each scFv to determine the different framework and complementarity determining regions of each sequenced PCR product.

Results

Expression of recombinant PRRSV nsp2 fragment protein

The expressed recombinant PRRSV nsp2 fragment protein was identified by SDS-PAGE to have an approximate size of 25 kDa. Protein size prediction based on amino acid sequence determined the fragment be 25.6 kDa in size which corresponded to SDS-PAGE. (Figure 2.1) The concentration of expressed protein was adjusted to10 µg/mL to coat 96 well polystyrene plates and 4 mL immunotubes. The expressed recombinant pHUE and pUb-GFP was shown to be the appropriate size by SDS-PAGE and concentration was adjusted to a concentration of 20 µg/mL and coated onto the 96 well plates.

Identification of bacterial clones containing scFv insert

E. coli strain TG1s were successfully transformed with each of the nine phagemid as evidenced by growth on ampicillin containing media, this strain of bacteria does not contain an ampicillin resistant gene. Enzyme cleavage by NcoI and NotI identified those bacteria containing the phagemid vector with the scFv insert on a 1.5% agarose gel; which were clones A9, B1, B9 and F10. Bands appearing at the expected size of the phagemid vector with insert (approximately 5.2 kb) were suspected of containing scFv coding insert. (Figure 2.2) Analysis of suspected clones by PCR utilizing the primers LMB3 and gIII identified the remaining clones containing scFv insert – A8, A11, D2, F9 and G3. (Figure 2.4) PCR products of the nine scFv were sent to ACTG, Inc., sequencing with subsequent analysis by Vector NTI software. (Table 2.2)

Selection and Production of scFv specific to recombinant PRRSV nsp2 fragment protein

Clones identified as containing the scFv insert were amplified by phage rescue and panned against the recombinant PRRSV nsp2 fragment protein coated to immunotubes. The eluted phage displayed scFv titers showed varying concentrations after each selection. (Table 2.1) The sequences of the nine scFv were aligned by Vector NTI and analyzed for Ig structure by IMGT/V Quest tool at http://www.imgt.org/IMGT_vquest/vquest. The analysis revealed that all but one scFv (D2) was of the conical familyV_H3, supporting previous reports of pig Igs being only of this family. The D2 scFv was determined to be of the family IGKV1. Alignment of all scFv as displayed in Table 2.2 show that A8, A11, B1 and possibly D2 are from the same V_H-V_L sequence insert, because of their identical amino acid sequence. The scFv A9 and F10 are also identical to one another with the remaining B9, F9 and G3 also sharing many of the same amino acid sequences however differing in four of the CDR3 sequences.

Western Blot Analysis

The Western Blot analysis to determine if the scFv specifically binds the recombinant PRRSV nsp2 fragment protein, human ubiquitin or the three synthetic peptides were inconclusive. See Figure 2.1 for peptide sequences. Bands that were faintly visible were determined to be non-specific binding of the secondary anti-actin HRP. The analysis is included only as showing a technique performed. (Figure 2.5)

ELISA

The initial ELISAs were performed to identify if scFv produced by panning were binding to the nsp2 fragment. The final amplifications (round 3 panning) of the different scFvs were serial diluted and assayed against the recombinant PRRSV nsp2 fragment protein. (Figure 2.7) The analysis showed the dilutional effect of phage displayed scFv binding to the nsp2 fragment and that there was a difference in the concentration of the scFv, with A8 being the lowest produced antibody fragment. The ELISA against the ubiquitin-GFP fusion protein and human ubiquitin revealed positive binding as determined by development of color and OD readings above background secondary control. (Figure 2.8) The average of two OD readings of each of the nine scFv antibodies were greater than 1.000 with the exception of A8, which was slightly greater than secondary antibody ODs; Blank wells had OD readings of <0.1000 and anti-M13 secondary antibody had average OD readings of 0.2408 for ubiquitin-GFP and 0.1047 for human ubiquitin coated wells. These results indicate that the nine phage displayed scFv recognize ubiquitin and are not specific for the recombinant PRRSV nsp2 fragment protein.

Immunoflourescent Assays of PRRSV Infected MARC-145 cells

MARC-145 cells were successfully infected with the PRRSV infectious clone pCMV-S-P129-1bMCS2-RFP within 48 hours of inoculation of virus. The individual wells for each of the nine phage display scFv did not show positive green fluorescence which signifies that the phage displayed scFv antibody fragments did not recognize the viral produced nsp2 protein. (Figure 2.9, D and E) Positive results were seen with purified SDOW17 monoclonal antibody which is specific to the PRRSV-N protein. (Figure 2.9, A-C) Negative controls with secondary antibodies showed no non-specific binding of either mouse anti-M13 or goat anti-mouse IgG (H+L) FITC. (Figure 2.9, F and G)

Discussion

Phage Display technology enables the researcher of today to produce antibodies unlike those produced by the accepted method of monoclonal antibody production. The methods developed by Georges Kohler and Roger Milstein in 1975 virtually changed the world of science.[99] The technique was developed with the knowledge of how antibodies were produced by mammals and using vaccination to manipulate the mouse immune system. It was known early on that an immune system responds to foreign antigen and that lymphocytes played an integral part in the response. Through investigations the B lymphocyte was found to be the antibody factories for mammalian systems and that each stimulated B cell was clonal and only produced an antibody recognizing a specific area of an antigen. The methods that Kohler and Milstein developed were profound in the fact that produced antibodies could be manufactured and be available for diagnostic testing, vaccine development and/or emerging disease investigation.[100] Since that time antibodies to a plethora of antigens have been developed by this system. However, the method is not without shortcomings. The limitations of monoclonal antibody production is the use of the mouse system, some antigens are not immunogenic to mice, antibodies produced are murine, some antigens may be toxic to mice and the time and effort to produce one antibody may take months to years are a few problems encountered in the past. The use of an animal system requires special areas for housing, handling and care which could be cost prohibitive.[71,82,101] Producing an antibody by screening a phage display library can bypass many of these roadblocks. The technique has developed libraries for a number of species, for example the small cloned library utilized by this study was developed from harvested pig spleens by the laboratory of Dr. Robert Aitken.[102-105] A phage display library potentially could contain an entire B cell repertoire from a particular species or be developed for an individual for specialized medical treatment.[82,106,107]

In this study, phage display techniques were utilized to screen cDNA cloned V_H and V_L sequences amplified from harvested spleens of pigs going to slaughter.[105] Initial screening was performed by Dr. Carol Wyatt while on a visit to Dr. Aitken's laboratory. It was the desire of researchers here at Kansas State University College of Veterinary Medicine to identify an antibody specific to a fragment of the PRRSV nsp2 protein. A recombinant nsp2 fragment protein had been developed in the past which was recognized by sera from PRRSV infected pigs; therefore, pigs produced antibodies to this particular area of the PRRSV nsp2 protein. 41] The nsp2 protein of the PRRSV is unique - deletions, mutations and insertions have been found within the middle area of the protein and virus replication continues and in some cases may enhance virulence of the virus.[59,108,109] PRRSV has plagued the pork producing community for decades and has cost millions of dollars in production

37

loss.[110] Several researchers have produced infectious clones with fluorescent marker inserts within this area of the PRRSV nsp2 as vaccine candidates.[1,48,55] Therefore, an antibody specific to this area that could be produced in large amounts would aid in the development of diagnostic tests to recognize those animals vaccinated from infected. Diagnostic tests that measure the progression of disease would be beneficial in determining the status of the herd or individual pig. By mapping the seroconversion time of antibodies that detect a particular epitope of the nsp2 protein would help to determine the progression and convalescence or carrier status of the pig.[45] The recombinant PRRSV nsp2 protein is a fusion protein with ubiquitin and contains a 6xHis tag, which aids in the purification of the protein. Dr. Wyatt's screening identified nine possible antibody fragments that bound to the recombinant nsp2 protein. Upon her return to Kansas, the nine phagemid cDNA vector samples were sent from the laboratory of Dr. Robert Aitken, Glasgow, Scotland in July, 2008. The nine samples were in microcentrifuge tubes with the labels – A8, A9, A11, B1, B9, D2, F9, F10, and G3, with approximately 50 µL of liquid in each tube. Information accompanying the samples stated that all of the phagemid clones were isolated from grown bacterial clones with phagemid containing V_H-linker-V_L insert after two to three rounds of panning. PCR was performed to verify that each sample phagemid contained the VH-liner-VL insert. Primers labeled LMB3 (5'-caggaaacagctatgac-3') and g3p (5'-ccctcatagttagcgtaacg-3') were utilized to amplify the insert. Gel electrophoresis of the PCR product showed bands of each clone to be approximately 1kb, with the exception of the D2 clone showing multiple bands. Clones B9 and F9 were not successfully amplified. Restriction enzyme fingerprinting of the PCR clonal product by BstN1 showed closely related genes (Figure A.2); however, sequence analysis confirmed variability within areas of the sequence for six of the nine clones. Partial sequence was attained on clone D2, clone B9 and F9 were not sequenced. (Figure A.3 and A.4) Ubiquitin screening by ELISA was not carried out prior to shipment of the phagemid vectors.

The *E. coli* strain TG1 was transformed by electroporation with the nine different phagemid DNA. Growth was sparse on the first two electroporation events and recovery of clones containing insert carrying phagemid DNA were not easily identified; therefore a total of four electroporation events were done to recover all the phagemid DNA with V_H-V_L inserts. Identification of inserts was difficult for this researcher. Several attempts at growing selected isolated colonies and restriction enzyme digestion was not successful. After further investigation, concentration of DNA was adjusted and digestion for a few clones yielded evidence of V_H-V_L inserts. (Figure 2.2) Gel Electrophoresis of purified phagemid DNA demonstrated that some of the clones did indeed contain the phagemid DNA and insert; a speculation based on the size of bands observed on the gel. The phagemid vector, pHEN is approximately 4.2 kb in size, the V_H-V_L insert ranges from 700 to 1000 kb in size, which should produce

a band at 4.9-5.2 kb; therefore purified clones that showed a band at this particular size was suspected of incomplete restriction enzyme digestion and was evaluated with a different method to identify the insert. The primer set utilized by Dr. Aitken was ordered and PCR was performed as outlined previously. The two techniques enabled the identification phagemid-insert positive clones that were then panned against the target protein. (Figure 2.3)

    After the third panning and amplification of scFv display phage, experimental infection of MARC-145 cells did not show specific binding of the scFvs. (Figure 2.9) Because the antibody fragments showed specific binding by ELISA, a suspension that the site of binding was due to recognition of the fusion product, possibly at the juncture of the amino acid sequence for the ubiquitin protein and the nsp 2 fragment protein was hypothesized. (Figure 2.7) Synthetic peptides were produced that corresponded to this juncture of the recombinant PRRSV nsp2 fragment protein. However Western Blot analysis was problematic, high non-specific binding of secondary antibodies yielded inconclusive results and subsequent ELISA showed no binding. (Figure 2.5) (ELISA data not shown)   An expressed recombinant fusion protein of ubiquitin-Green Florescent Protein and a commercially produced human ubiquitin was coated onto 96 well plates and an ELISA was performed for each of the nine scFv. The addition of anti-M13 HRP detected phage displayed scFv binding to both ubiquitin containing wells. This information supports the ELISA performed with the synthetic peptides, the juncture is irrelevant to the scFv binding. However, no binding was seen with the ubiquitin on Western Blot suggesting the binding is conformational to the ubiquitin; the denaturing conditions of SDS-PAGE and Western Blots linearize proteins. Because binding was detected in both it was apparent that the scFvs selected were not specific for the PRRSV nsp2 fragment protein but for the ubiquitin portion of the fusion protein.

    Sequence analysis of the PCR products for each of the scFv revealed that six of the nine possessed the same amino acid sequence and therefore the same scFv. Alignment of all scFv as displayed in Table 2.2 show that A8, A11, B1 and possibly D2 are from the same V_H-V_L sequence insert and that A9 and F10 are the same as well. The sequence of each scFv was analyzed using the IMGT/V Blast tool to identify specific areas of the antibody and determine if they were of a particular Ig family. Published studies reported that pig Igs are structurally within one family – identical to the human V_H3 family of Igs.[111,112] Results obtained on eight of the scFv were indeed classified by IMGT as V_H3 Ig fragments except the D2 scFv, which was determined to be of the family IGKV1. The information leads one to consider the possibility of scFv D2 was a linkage of two V_L fragments. The remaining scFv; B9, F9 and G3 shared many same amino acid sequences with A9 and F10; however, the CDR3 sequences differed by four amino acids. Sequence analyses provided by Dr. Aitken were significantly different in size and in the sequences of the CDR2 and onward of each of the scFv produced by subsequent panning.

The difference in analysis could possibly be due to the different analysis tools utilized to determine the framework and CDRs of each of the scFv. Published studies do report changes in recovered scFv with each panning event. Each time the phagemid-insert is manipulated there is a chance that a selection of higher or lower affinity antibody fragments or the possibility of errors in transcription or translation causing mutations in amino acid sequences, even the loss of insert has occurred.[64]

In Conclusion, phage display technology is a valuable tool in the identification and production of antibody fragments. This study demonstrates that the techniques are relatively easy to perform but there are several steps that build to produce the end product – a fully functional antibody fragment that identifies a specific epitope of a target antigen. However, one must be aware of the pitfalls that can occur when target antigens may contain area which do not contain the desired epitopes; such as the ubiquitin in the recombinant PRRSV nsp2 fragment protein. Future projects will keep these lessons learned at the forefront of the investigation. Intense screening of irrelevant proteins contained as fusion products to target antigens will always be considered a potential target. The aim of this study was to produced previously selected scFv specific to the nsp2 PRRSV protein; however it was found that all selections recognized areas of the ubiquitin within in the recombinant PRRSV nsp2 fragment protein.

Figure 2.1 Schematic drawing of PRRSV nsp2 protein.
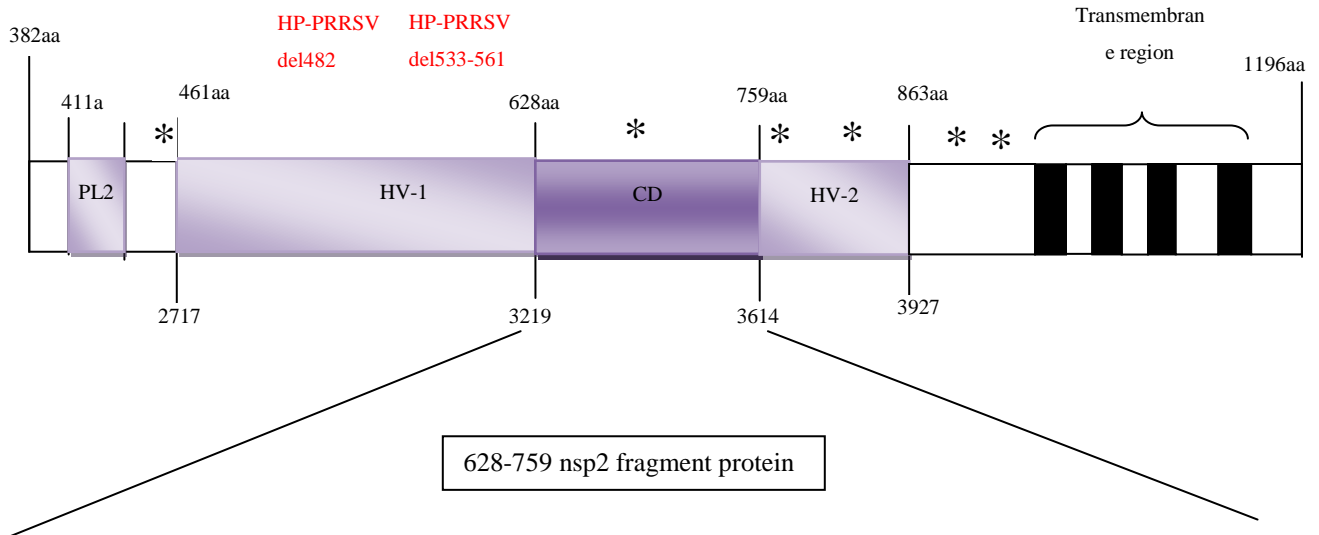
MGSS*HHHHHH* SSGLVPRGSH MQIFVKTLTG KTITLEVEPS DTIENVKAKIQDKEGIPPDQ
QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGGRPKYSAQAIIDLGG PCSGHLQREK
EACLRIMREA CDAAKLSDPA TQEWLSRMWDRVDMLTWRNT SAYQAFRTLD GRFGFLPKMI
LETPPPYPCG FVMLPHTPAPSVSAESDLTI GSVATEDIPR ILGKIENTGE

Peptide 1: LHLVLRLRGGRPKYSAQAII
Peptide 2: LHLVLRLRGGDLGGPCSGHL
Peptide 3: SVSAESDLTIGSVATEDIPRILGKIENTGE

Figure 2.1: Recombinant PRRSV nsp2 fragment protein amino acid sequence (634-769) containing a *6xHis tag* and pHue ubiquitin (labeled red) sequence upstream from the nsp2 fragment sequence. Peptide 1 corresponds to the junction of the ubiquitin-nsp2 fragment sequence. Peptide 2 is a discontinuous sequence of the last seven amino acids of ubiquitin, skipping the next 11 amino acids, with the next eleven sequences of the nsp2 fragment. Peptide 3 is the last 30 amino acids of the nsp2 fragment which contains amino acid homology to a reported B-cell linear epitope (PRILGKIEN).[44]

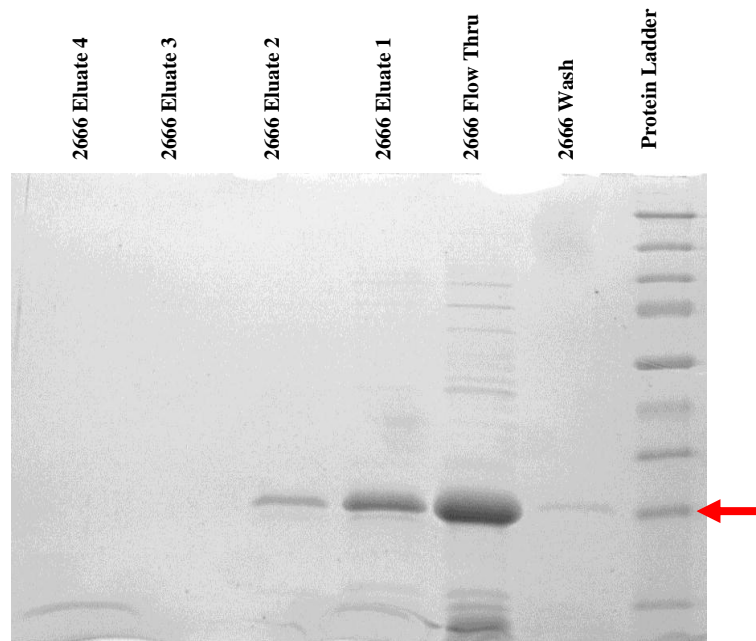**Figure 2.1: SDS-PAGE of Expressed recombinant PRRSV nsp2 fragment protein.**

Figure 2.1: 12% SDS-PAGE gel of expressed recombinant PRRSV nsp2 fragment protein showed a distinctive band at approximately 25 kDa sizes (←) which corresponds to predicted size of fusion protein.

Figure 2.2: Agarose Gel Electrophoresis of restriction endonuclease digestion of purified Phagemid DNA from selected clon
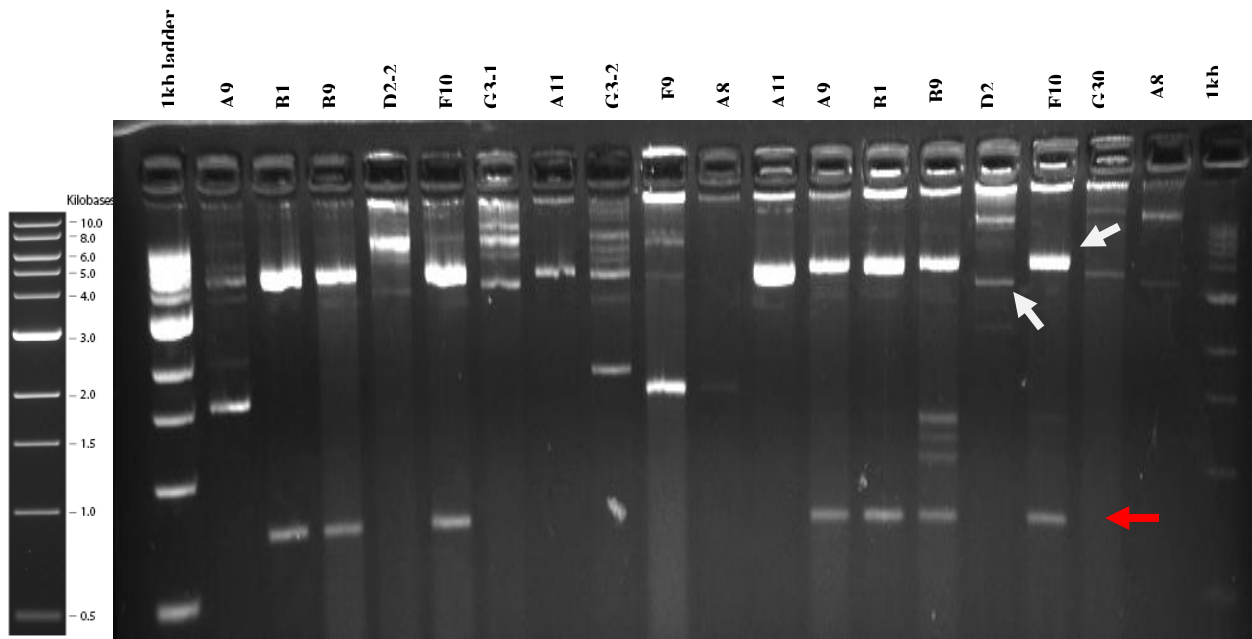

Figure 2.2: Gel electrophoresis of purified phagemid DNA from selected clones after restrictive endonuclease digestion with NotI and NcoI restriction enzymes. Selected clones with VH-linker-VL insert are predicted to be approximately 1.0 kb in size. (red arrow) Clones without insert estimated to be approximately 4.2 kb or with insert but partial to no digestion estimated to be approximately 5.2 kb (white arrows).

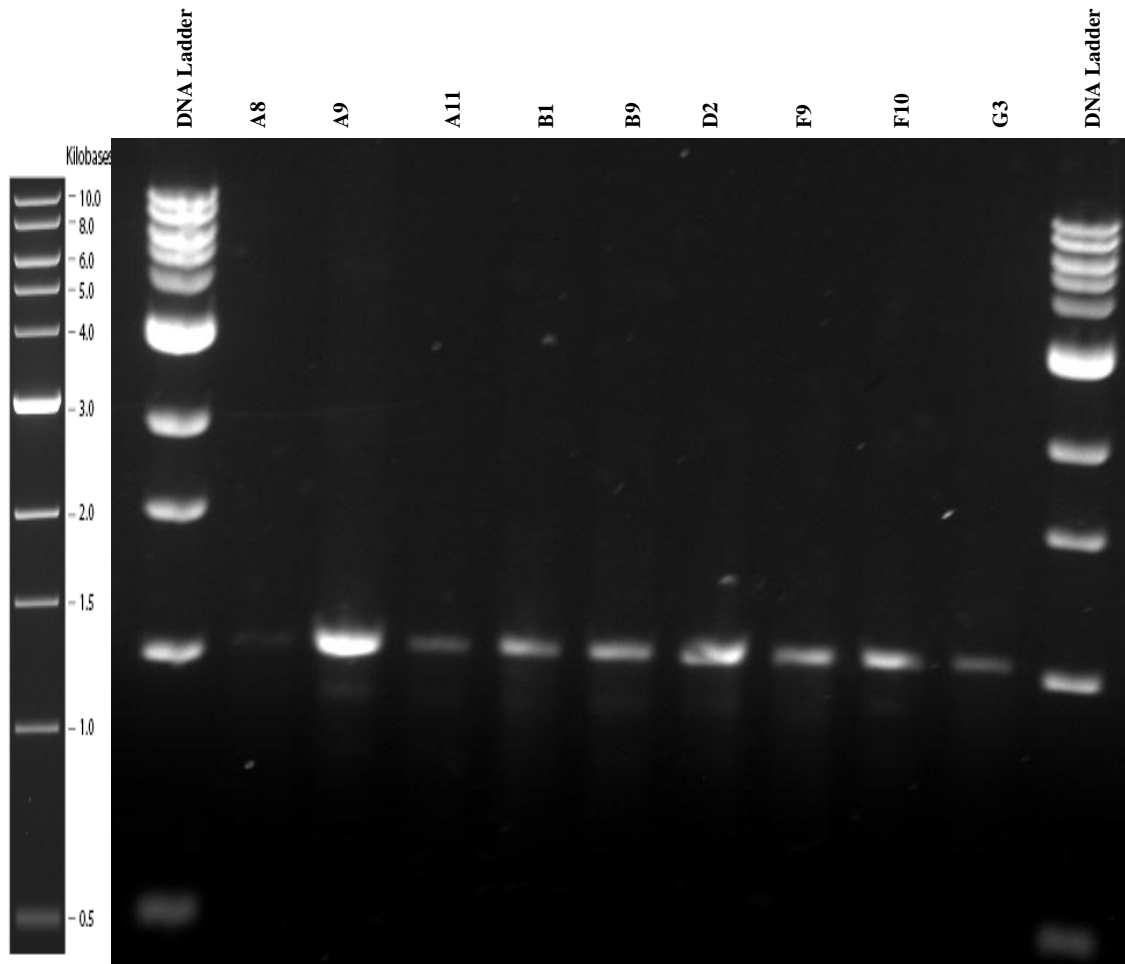**Figure 2.4: Amplified DNA insert for each scFv.**

Figure 2.3: Gel electrophoresis of PCR amplified purified DNA from selected bacterial clones containing phagemid-insert vector for the nine selected scFv.

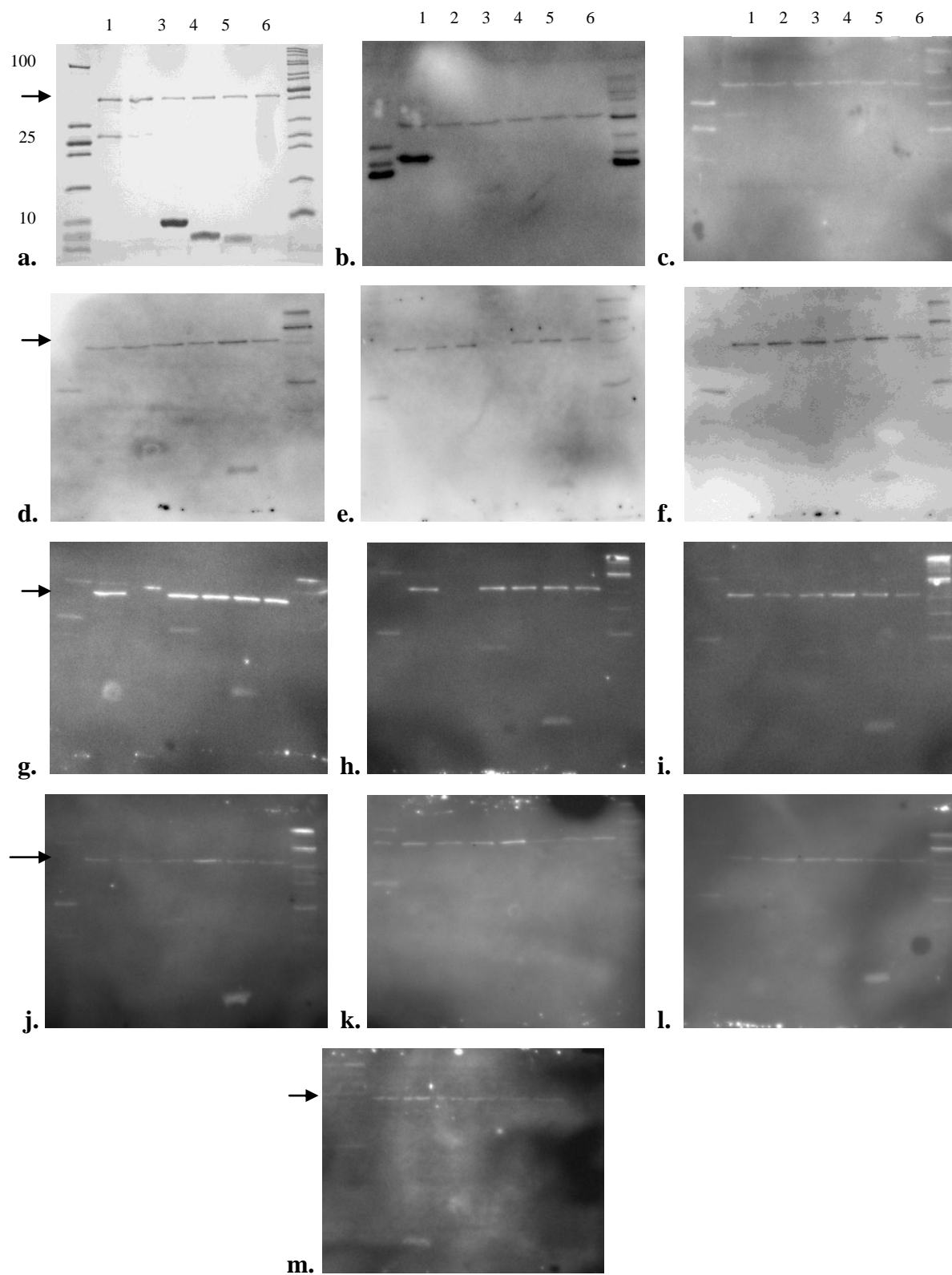# Figure 2.5 SDS-PAGE and Western Blot of Phage Displayed scFv.

Figure 2.5: a) SDS-PAGE of recombinant PRRSV nsp2fragment protein – lane 1, trypsin treated nsp2 fragment protein – lane 2, Ubiquitin – lane 3, Peptide 1 – lane 4, Peptide 2 – lane 5, Peptide 3 – lane 6. b) Western Blot (WB) of positive control – pooled pig sera with positive band in lane 1. c) WB of negative control – pooled pig sera showing no bands. d) WB of scFv A8 showing possible positive band for Peptide 2 – lane 5; however background staining could not rule out possible non-specific binding. e) WB of scFv A9. f) WB of scFv A11. g) WB of scFv B1. h) WB of scFv B9. i) WB of scFv. j) WB of scFv F9. k) WB of scFv F10. l) WB of scFv G3. m) Negative Control of secondary antibody for anti-sheep IgG FITC showing possible positive band to ubiquitin – lane 3. Black arrow denotes β-actin loading control at a MW of 42.

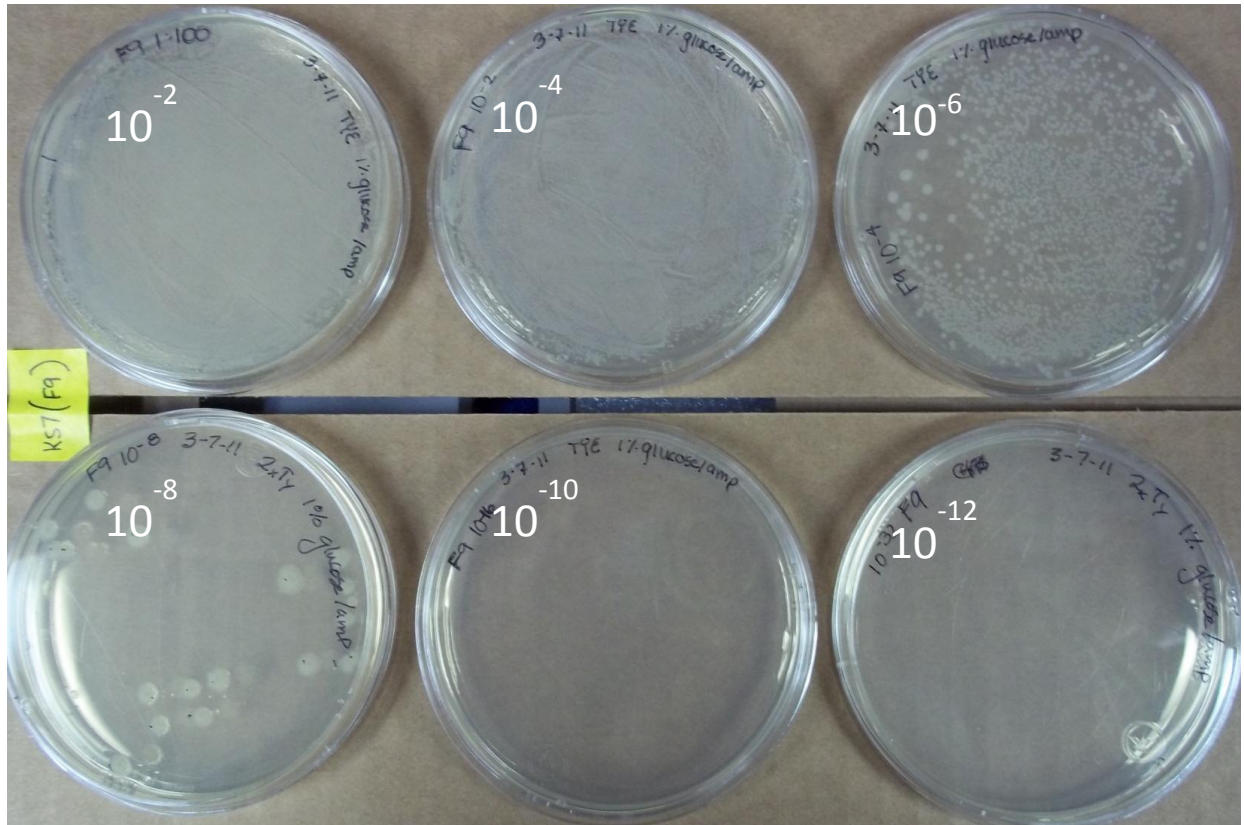**Figure 2.6 Phage titer culture plates for A8 scFv**

Figure 2.6: Representative culture plates (2xTy with 100 μg/mL of Ampicillin and 1% glucose) showing titer of eluted phage from F9 scFv containing clones.

Table 2.1: Titer Concentration of phage

scFv Clone	Selection 1 Titer	Selection 2 Titer	Selection 3 Titer	Selection 4 Titer
A8	$>1.0 \times 10^6$	$>1.0 \times 10^4$	2.0×10^4	3.0×10^8
A9	3.3×10^5	$>1.0 \times 10^4$	1.0×10^{12}	3.7×10^9
A11	2.6×10^{14}	1.1×10^6	1.0×10^{12}	3.9×10^9
B1	2.1×10^{14}	4.1×10^6	1.0×10^{14}	4.0×10^8
B9	3.5×10^{14}	5.4×10^5	1.0×10^{13}	1.1×10^9
D2	3.3×10^{14}	6.4×10^5	1.0×10^{14}	1.3×10^{10}
F9	1.5×10^9	300	1.0×10^{13}	4.0×10^8
F10	5.1×10^9	15	1.0×10^{14}	7.6×10^9
G3	6.0×10^9	$>10 \times 10^4$	1.0×10^{15}	1.4×10^9

Table 2.1: Titer Concentration of eluted phage after each round of selection grown on 2xTY with 100 µg/mL of Ampicillin and 1% glucose. Phage titers were determined by diluting 1µL of eluted phage to 100 µL PBS (10^{-2}), then 1µL of the 10^{-2} dilution to 100 µL PBS, and so on until there were 6 total dilutions for each of the scFv displayed eluted phage.

Table 2.2: Sequence analysis and IMGT alignment of scFv

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              FR1-IMGT              CDR1-IMGT      FR2-IMGT      CDR2-IMGT         FR3-IMGT                CDR3-IMGT
              (1-26)                (27-38)        (39-55)       (56-65)          (66-104)                (105-113)

     1    10        20        30        40        50        60        70        80        90        100
     .........|.........|......  ...|.........  .|.........|.....  ..  ..|.....  ....|.........|.........|.........|....
A8   DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTPNT
A9   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVST IG GS..GSST YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKGANYFDY
A11  DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTPNT
B1   DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTPNT
B9   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVSS IA SH..GSRT *YADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKRTSRFDY
D2   DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTP
F9   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVST INTN...GDNT NYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKGAGTFDY
F10  EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVST IG GS..GSST YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKGANYFDY
G3   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVSS IG GS..GTYT YYADSVK.GRFTISRDNSKNTLYLQMNSLGAEDAAVYYC AKSTSSFDY


A8   DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTPNT
A11  DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTPNT
B1   DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTPNT
D2   DIQMTQSPSSLSASVGDRVTITCRAS QSI......SSY LNWYQQKPGKAPKLLIY AA .......S SLQSGVP.SRFSGSG..SGTDFTLTISSLQPEDFATYYC QQSYSTP

A9   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVST IG GS..GSST YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKGANYFDY
F10  EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVST IG GS..GSST YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKGANYFDY

B9   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVSS IA SH..GSRT *YADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKRTSRFDY
F9   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVST INTN...GDNT NYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AKGAGTFDY
G3   EVQLLESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVSS IG GS..GTYT YYADSVK.GRFTISRDNSKNTLYLQMNSLGAEDAAVYYC AKSTSSFDY
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Table 2.2: Sequence analysis of PCR products by ACTG, Inc with LMB3 and gIII primer set. Analysis performed with Vector NTI software. IMGT/V bioinformatics tool determined framework and complementarity-determining regions of each insert sequence.

Figure 2.7: Positive binding to recombinant PRRSV nsp2 fragment protein of serial diluted scFv.
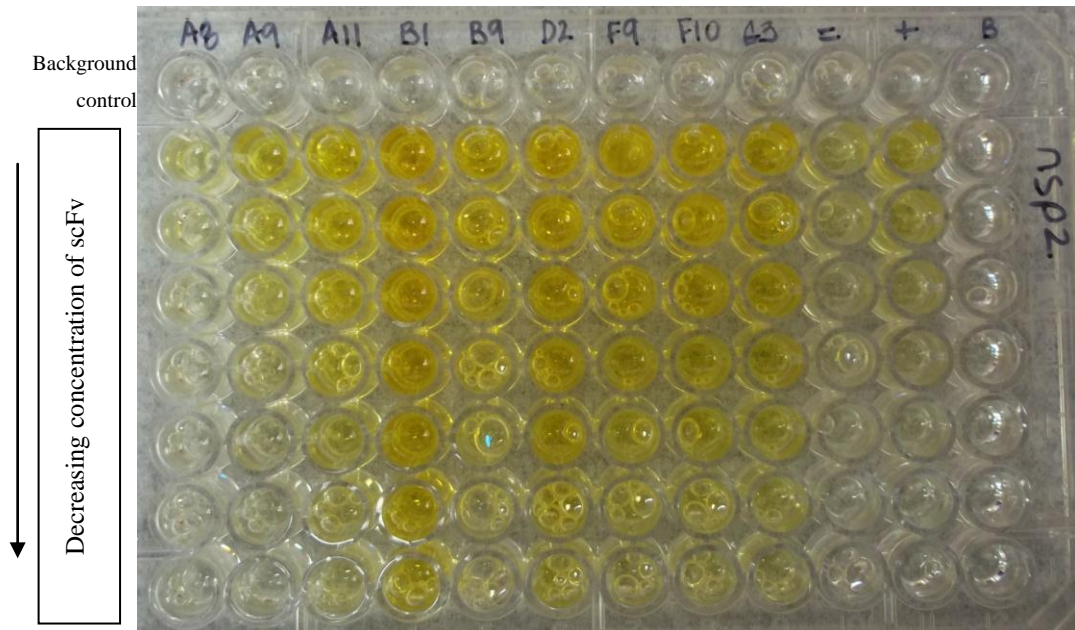

Figure 2.7: ELISA of serial diluted scFv against a recombinant PRRSV nsp2
fragment protein coated 96 well plates. The first (A1-A12) do not contain the nsp2
fragment protein is the background control for bound scFv. Pooled sera from infected
and non-infected pigs were added for binding controls. Blank wells B12-D12
contained the secondary anti-M13 HRP and E12-H12 contained anti-pig IgG HRP
and functions as the secondary antibody background control.

Figure 2.8: ELISA of scFv screened for specific binding to recombinant GFP-ubiquitin fusion protein and human ubiquitin.

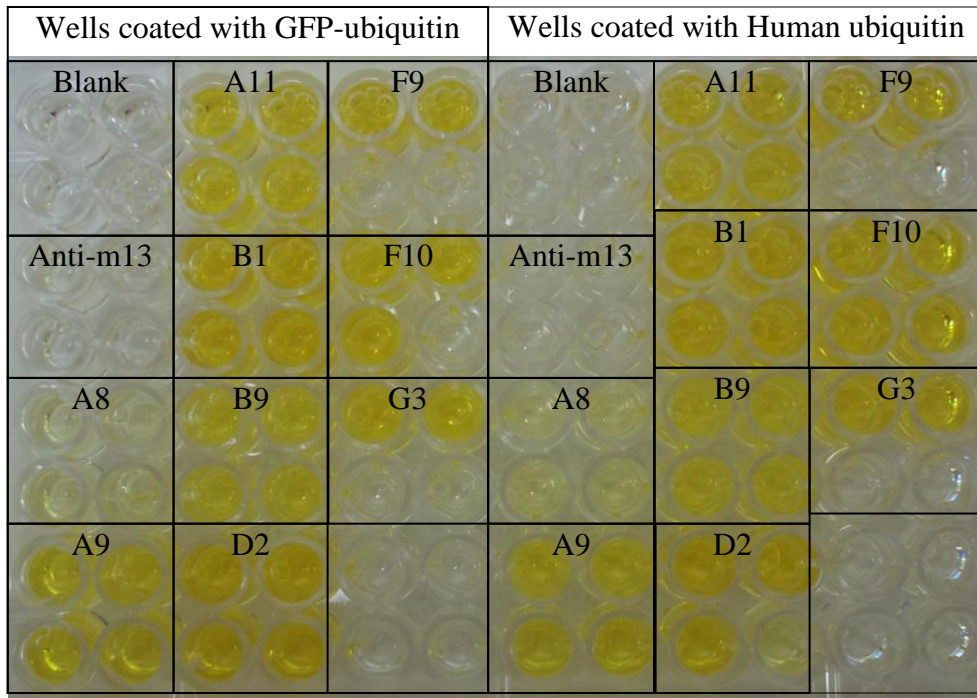| Wells coated with GFP-ubiquitin			Wells coated with Human ubiquitin		
Blank	A11	F9	Blank	A11	F9
Anti-m13	B1	F10	Anti-m13	B1	F10
A8	B9	G3	A8	B9	G3
A9	D2		A9	D2	

Figure 2.8: ELISA showing positive binding of scFvs to wells coated with GFP-ubiquitin (Row 1-6) and human ubiquitin (Row 7-12).

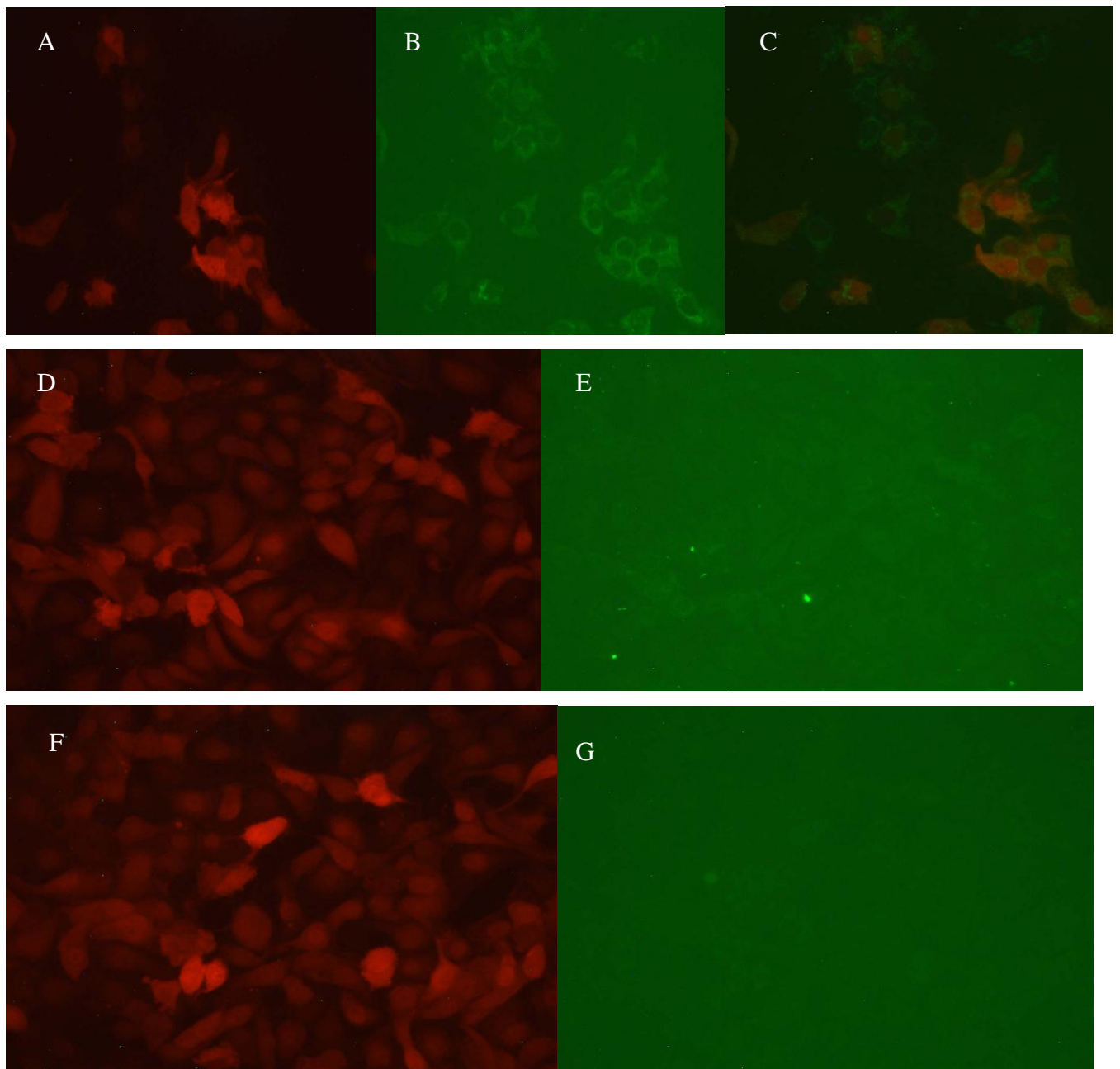**Figure 2.9: IFA of PRRSV infected MARC-145 cells.**

Figure 2.9: IFA of PRRSV infected MARC-145 cells. A) Red fluorescence indicating viral replication of PRRSV pCMV-S-P129-1bMCS2-RFP infectious clone. B) SDOW17 antibody labeled cells indicating production of PRRSV-N protein serves as positive control for infection and secondary antibody performance. C) Merge of A and B. D and E) Representative picture of assay for specific binding of scFv to infected cells. All wells assayed showed positive viral production. No green fluorescence detected from any wells that scFv were added indicating no recognition of viral produced nsp2 protein, exposure time increased by half in order to show cells. F and G) Background control of mouse anti-M13 with secondary goat anti-mouse IgG (H+L) FITC antibody indicating no non-specific binding detected.

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Supplemental Data

Figure 0.1 Gel electrophoresis of PCR product from purified phagemid DNA from bacterial clones.
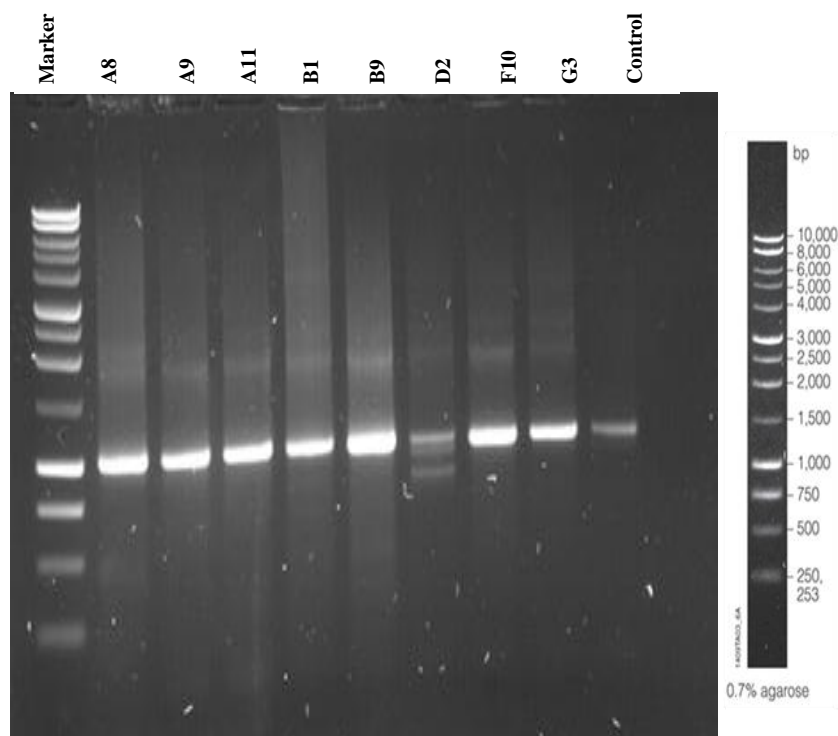

Figure A.1: PCR amplification of eight of the nine clones performed by Aitken laboratory shows bands approximately 1kb in length, expected length of VH-linker-VL insert was supported. D2 clone showed multiple length amplification products.
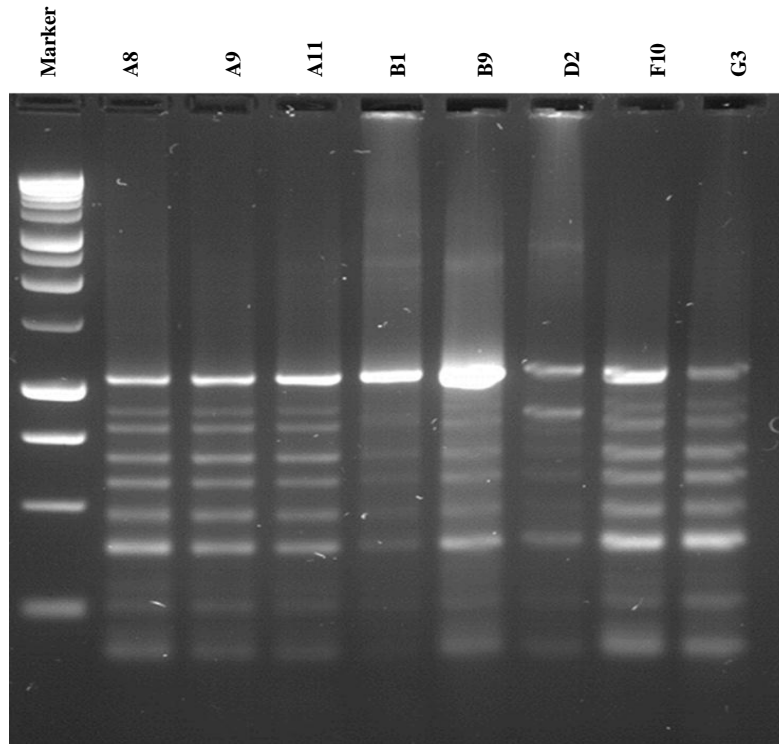
Figure A.2 Restriction Enzyme Fingerprinting.

Figure A.2: Fingerprinting results with restriction enzyme BstN1 showing similar size fragments of DNA.

Figure A.3: Sequence analysis of scFv

```
>A8_complete
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TCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTATTGTGCGAAAGGTGGTGGTAGT
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>A9_complete
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TTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGTGGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGAC
CCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAAC
CAGGGAAAGCCCCTAAGCTCCTGATCTATAGTGCATCCTCTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACC
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GGCCGCACATCATCATCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAG
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>A11_LMB3
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```

No scFv match at GenBank.

>B1_complete
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ATGGCCGAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGC
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GGCCGCACATCATCATCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAG
```

Note: stop codon in VH domain

>D2_LMB3 (partial)
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CATGAGCTGGGTCCGCCAGGGCTCC
```

>D2_gIII (partial)
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```

Note: sequencing ambiguities in gIII data fail to create intact reading frame.

>F10_complete
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CCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAAC
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>G3_complete
```
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```

Figure A.3: Sequence analysis of recovered scFv with comments.

Figure A.4 Alignment of sequences.

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A8     1    MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTISGNGGAT
A9     1    MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSISSNGGYT
B1     1    MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTIRRAG-FT
F10    1    MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTIGGSGSST
G3     1    MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIGGSGTYT
D2     1    MAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQGS...


A8     61   YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGGGSFDYWGQGTLVTVSSGG
A9     61   TYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGAGSFDYWGQGTLVTVSSGG
B1     61   SYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKRGLRFDYWGQGTLVTVSSGG
F10    61   YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKGANYFDYWGQGTLVTVSSGG
G3     61   YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTSSFDYWGQGTLVTVSSGG


A8     121  GGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKL
A9     121  GGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKL
B1     121  GGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKL
F10    121  GGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKL
G3     121  GGSGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKL


A8     181  LIYSASDLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSNPTTFGQGTKVEI
A9     181  LIYSASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSNSNPSTFGQGTKVEI
B1     181  LIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTPNTFGQGTKVEI
F10    181  LIYTASALQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQANTYPATFGQGTKVEI
G3     181  LIYNASYLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSAYAPYTFGQGTKVEI


A8     241  KRAAAHHHHHHGAAEQKLISEEDLNGAA-
A9     241  KRAAAHHHHHHGAAEQKLISEEDLNGAA-
B1     241  KRAAAHHHHHHGAAEQKLISEEDLNGAA-
F10    241  KRAAAHHHHHHGAAEQKLISEEDLNGAA-
G3     241  KRAAAHHHHHHGAAEQKLISEEDLNGAA-
```

Figure A.4: Sequence analysis results from six of the nine clones. Amino acid translation *in silico* with manual alignment of the six sequenced clones. Highlighted areas denote sequence variability.