Molecular Breeding of Porcine Circovirus Type 2 by Synthetic DNA Shuffling

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Abstract

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, single-stranded DNA virus that causes disease in pigs and is an economically important pathogen affecting pig populations worldwide. PCV2 contains two major open reading frames (ORF): ORF1 encodes two replicase proteins and ORF2 encodes the immunogenic capsid protein. There are three genotypes of PCV2 (PCV2a, PCV2b, and PCV2c), but vaccines available for PCV2 infection are only targeted against PCV2a. The objective of this thesis was to create viable chimeric PCV2 viruses with an ORF2 displaying genetic diversity from all PCV2 genotypes by synthetic DNA shuffling.

Variation was identified at 55 amino acid positions in the ORF2 gene among 853 PCV2 capsid gene sequences available in the GenBank database. Degenerate oligonucleotide primers spanning ORF2 were synthesized to contain this naturally observed sequence diversity. Sets of overlapping oligonucleotide primers were fused together using overlap extension PCR to create full-length shuffled ORF2 sequences. The shuffled library of the ORF2 genes was subsequently cloned into the genomic backbone of a wildtype PCV2a infectious DNA clone and transfected into porcine kidney cells (PK-15). After transfection and infection of PK-15 cells, viability of chimeric viruses was screened by immunofluorescence assay (IFA) using anti-PCV2 Rep antibodies. PCR was used to amplify the genomes of viable shuffled viruses from infected cells. PCV2 viruses containing an ORF2 displaying genetic diversity from PCV2a, PCV2b, and PCV2c were isolated *in vitro*. These shuffled PCV2 viruses may be used as potential candidates for a broadly-protective PCV2 vaccine, although additional studies are warranted to determine *in vivo* infectivity and pathogenicity.

I dedicate this thesis to my loving and incredibly supportive parents, Todd and Jeannette Smith.

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Abbreviations

AASV	American Association of Swine Veterinarians
AAV	Adeno-associated virus
BFDV	Beak and feather disease virus
bp	Base pair
DEN	Dengue virus
DNA	Deoxyribonucleic acid
dpi	Days post infection
ELISA	Enzyme linked immunosorbant assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HA	Hemagglutinin
IFA	Immunofluorescence assay
IHC	Immunohistochemistry
ISH	*in situ* hybridization
kb	Kilobases
MEM	Minimum essential medium
MLV	Murine leukemia virus
nt	Nucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
pBSK II	pBluescript II SK (+) vector
PCR	Polymerase chain reaction
PCV	Porcine circovirus
PCV1	Porcine circovirus type 1
PCV2	Porcine circovirus type 2
PCV2-S	Full-length shuffled PCV2 virus
PCV2-S-HA	Full-length shuffled PCV2 virus with HA tag addition
PCVAD	Porcine circovirus-associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PK-15	Porcine kidney cell line
PMWS	Postweaning multisystemic wasting syndrome
PPV	Porcine parvovirus
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
SIV	Swine influenza virus
USDA	United States Department of Agriculture
VBI	Virginia Bioinformatics Institute, Blacksburg, VA

General Introduction

Porcine circovirus (PCV), a member in the family *Circoviridae*, is a non-enveloped, single-stranded DNA virus. PCV is one of the smallest known animal viruses of approximately 1.7 kb in length with two main open reading frames (ORF1 and ORF2). ORF1 encodes for replicase proteins while ORF2 encodes for the immunogenic capsid protein. There are two phenotypically different but genetically related types of PCV documented to date, PCV type 1 and PCV type 2 (PCV1 and PCV2). PCV1 was first discovered in 1974 as a contaminant of the porcine kidney cell line, PK-15, and is considered to be non-pathogenic. PCV2, on the other hand, is the primary causative agent of porcine circovirus-associated disease (PCVAD) which includes postweaning multisystemic wasting syndrome (PMWS). Currently, PCV2 is one of the most economically important viral pathogens affecting pig populations worldwide.

There are three distinct genotypes of PCV2 documented to date: PCV2a, PCV2b, and PCV2c. PCV2a isolates have been predominantly of North American origin, while the PCV2b genotype has been found primarily throughout Europe and Asia. Since 2005, PCV2b has become more prevalent in the United States along with an increased severity of PCVAD. A third genotype, PCV2c, was isolated from Denmark in the 1980's, but only from non-diseased herds. The distinct PCV2 genotypes arise from nucleotide sequence diversity in the capsid while the replicase remains highly conserved between all genotypes.

There are four commercially available vaccines for PCV2 and the control of PCVAD; each based upon the PCV2a genotype. All are killed or recombinant subunit vaccines and have been successful in reducing mortality in piglets caused by PCV2 throughout North America, South America, and Europe. However, since PCV2b has become more prevalent worldwide and as viruses continue to evolve, it is unknown whether the current PCV2a-based vaccines can

provide complete cross-protection against PCV2b and other emerging strains. Therefore, it is important to develop a broadly-protective vaccine that will take into consideration the heterogeneity of all known PCV2 genotypes, as well as potential mutations arising from vaccine selection pressure.

Molecular breeding-directed evolution, also known as DNA shuffling, is an accelerated *in vitro* evolution process that produces recombinants from a group of related parental genes which can be screened and selected for improved characteristics. There are two primary methods for molecular breeding of viruses: multi-gene shuffling and synthetic DNA shuffling. The multi-gene shuffling method consists of a pool of related parental genes which are fragmented into short DNA segments and allowed to randomly reassemble using PCR technology. Synthetic DNA shuffling involves synthetic degenerate oligonucleotides in which every amino acid from a set of related parental gene sequences is allowed to recombine independently of every other amino acid. With synthetic DNA shuffling, starting parental genes are unnecessary and specific mutations can be incorporated to produce chimeras that would not have been created through the multi-gene shuffling method. The key feature of molecular breeding in vaccine development is that the resulting chimeras can be selected for improved cross-reactivity and enhanced immunogenicity against multiple strains of a pathogen. The objective of this thesis is to employ the technique of synthetic DNA shuffling to produce viable recombinant PCV2 viruses containing a capsid gene displaying genetic diversity from all known PCV2 genotypes.

Chapter 1

Literature Review

Porcine circovirus

Porcine circovirus (PCV) is a small, non-enveloped, single-stranded DNA virus. PCV has a circular genome surrounded by an icosahedral capsid of approximately 17 nm in diameter (7, 128). PCV is a member of the family *Circoviridae* (95) which is divided into two genera: *Gyrovirus*, consisting of the chicken anemia virus, and *Circovirus*, containing PCV, beak and feather disease virus (BFDV), canary circovirus, goose circovirus, and pigeon circovirus (95, 108, 125).

PCV is one of the smallest known animal viruses (84) whose genome is approximately 1.7 kilobases (kb) in length consisting of two main open reading frames (ORF 1 and ORF2) (50) (Figure 1.1). ORF 1, which is 942 base pair (bp) in length on the positive sense strand, encodes two non-structural proteins, Rep and Rep', that are involved in viral replication (14). ORF 2, 702 bp in length, encodes for the immunogenic structural capsid protein (Cap) on the negative sense strand (79, 87). A third open reading frame, ORF3, was identified as a protein involved in host cell apoptosis (72), although others failed to reproduce the finding. The PCV genome contains a 111 bp origin of replication region between the capsid and replicase genes (80) and replicates via the rolling-circle method (15). The mechanisms of the PCV life cycle are not well understood, but because the virus is so small, PCV is highly dependent upon host cell machinery for replication and protein expression. The virus does require an actively replicating cell, specifically one that is in the S phase of cell cycle replication (130).

There are two phenotypically distinct types of PCV documented to date: PCV type 1 (PCV1) and PCV type 2 (PCV2) (5). PCV1, first discovered in 1974 as a contaminant of the porcine kidney cell line, PK-15, is considered to be non-pathogenic and the only non-pathogenic member of the *Circoviridae* family (6, 8, 129-131). In 1997, PCV2 was first described and found to be associated with a clinical disease in piglets known as postweaning multisystemic wasting syndrome (PMWS) in Canada (3, 32, 51, 70, 88). PCV1 is 1758 nucleotides (nt) in length while PCV2 is 1767 or 1768 nt and the two viruses share approximately 68-75% nt sequence identity (39, 128). The degree of nucleotide sequence identity in the replicase gene between PCV1 and PCV2 is about 83% while the capsid gene shares 67-70% sequence identity (79).

Even though PCV2 was not identified until 1997, the presence of PCV2 specific antibodies from archived serum samples can be traced back to 1969 in Belgium (114), 1970 in the United Kingdom (47), 1973 in Ireland (132), 1980 in Denmark (31), and 1985 in both Canada and Spain (77, 109). In 1986, it was determined that 77-95% of pigs in Berlin were seropositive for PCV but did not show any signs of disease (129). Following the identification of PCV2 and PMWS, PCV2 became the predominant circulating type (107). The prevalence of PCV2 in other affected countries such as Canada, Spain, Taiwan, and Australia range from 40 to 80% (40, 77, 109, 133). PCV2 has also been documented in the United States, Switzerland, Hungary, Japan, Korea, and countries of South America (7, 59, 62, 91). Both PCV1 and PCV2 are considered to be widespread in pig populations and found in both diseased and non-diseased herds worldwide (9).

PCV2 genotypes

There are three distinct genotypes of PCV2 documented to date: PCV2a, PCV2b, and PCV2c. The distinct PCV2 genotypes arise from nucleotide sequence diversity in the capsid while the replicase remains highly conserved between all known PCV2 genotypes. Phylogenetic trees for PCV2 are based upon the capsid gene since it is possible to reconstruct trees with similar topology when using the whole viral genome (91). There is approximately 93-95% sequence identity between PCV2a and PCV2b within the capsid gene and 91-95% sequence identity between PCV2c against PCV2a and PCV2b capsid at the nucleotide sequence level (31, 91). At the protein level, sequence identity is 97-100% for the replicase and 89-100% for the capsid protein among all genotypes (69). PCV2a can be further divided into five clusters and PCV2b can be divided into three clusters (91).

Prior to 2005, the principal circulating genotype throughout the North America was PCV2a, while PCV2b isolates were found primarily throughout Europe and China (17, 34). However, over the past eight years, PCV2b has become more prevalent in the United States and has been linked to more severe outbreaks of PCV2-related diseases (43). A third genotype, PCV2c, was isolated from Denmark in the 1980's, but only from non-diseased herds (31).

Porcine circovirus-associated disease (PCVAD)

History

PCV2 was first observed in association with PMWS in Canadian piglets in 1991 (4, 7, 51), while in the United Kingdom and Ireland the disease was first described in 1998 (7). In 2006, the term porcine circovirus-associated disease (PCVAD) was approved by the American Association of Swine Veterinarians (AASV) to include all clinical diseases arising from PCV2

infection (95). PCV2 antibodies are present in swine herds worldwide with up to 100% of individual pigs within those herds being positive, including herds in the United States (77, 100, 132). PCV2 has a high prevalence of infection, but morbidity rates are low since not all pigs with PCV2 develop a clinical disease. However, mortality rates due to PCVAD can be as high as 80% in some affected herds (25). Currently, PCV2 is one of the most economically important viral pathogens affecting pig populations worldwide, and PCVAD is considered to be an emerging disease with incidence continuing to increase (95).

Transmission and route of infection

Since PCV2 is non-enveloped and resistant to pH 3 and temperatures of 70ºC for 15 minutes (8), the virus is very stable in the environment and therefore easily transmissible in swine herds. The major route of transmission is through oro-nasal contact with infected feces, urine, or other pigs (11, 76). It has also been demonstrated that PCV2 can be vertically transmitted from sow to offspring, resulting in persistently infected piglets, but this route of transmission is uncommon (78, 103, 104, 124).

PCV2 infects 8 to 15 week-old piglets shortly following decay of maternal antibodies (81, 83). The incubation period of PCV2 ranges from 7 to 28 days with early clinical signs of fever, lethargy, weight loss, enlarged lymph nodes, dark-colored diarrhea, and jaundice (4, 12, 51, 70). PCV2 first infects the tonsils and lymph nodes of the head where viral replication begins (110). Following infection of B cells, the virus becomes widespread throughout the lymphatic, respiratory, urogenital, and gastrointestinal system and is found in most excretions and secretions such as feces, urine, semen (68, 82), oro-nasal swabs, colostrum (120), and blood (110, 121). Viremia is detected between 7 and 14 days postinoculation (dpi), and PCV2 DNA

can be detected up to 125 dpi in experimentally infected pigs (5, 9, 11, 53, 65, 76, 106, 112). Later clinical signs may include dyspnea, tachypnea, and anemia as well as more severe complications such as enteritis, pneumonia, reproductive failure, porcine dermatitis and nephropathy syndrome, neuropathy, and death (52, 56, 60, 66, 90).

Co-infections with other swine pathogens

Infection with PCV2 alone does not typically cause a clinical disease in swine, but co-infections with other pathogens such as swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), and other bacterial pathogens such as *Mycoplasma hypopneumoniae* increase the severity of PCVAD (2, 29, 76). PCV2 by itself causes disease in only about 1% of cases (30, 33, 45, 102) while PRRSV is the most commonly associated pathogen with PCV2 appearing in approximately 52% of PCVAD cases in the United States (108).

Syndromes

PCVAD can result in a variety of outcomes, ranging from a subclinical infection to severe clinical manifestations including weight loss, respiratory disease, reproductive failure, enteritis, dermatitis, and high mortality. Observation of histopathological lesions in the intestines, lungs, and lymphoid tissues along with the presence of PCV2 antigen can be used to distinguish between the different forms of PCVAD.

Subclinical PCVAD occurs when the PCV2 is present in the tissues, but no clinical signs are observed. The viral infection can be limited to one or two lymph nodes with minimal to no

lesions present in the tissue (93, 99). It has also been experimentally shown that subclinical PCVAD can arise from decreased vaccine efficacy (94).

The most significant and recognizable clinical manifestation of PCVAD is PMWS which typically affects 7 to 16 week-old piglets (4, 19, 119). Clinical signs of PMWS include weight loss, fever, lethargy, enlarged lymph nodes, dyspnea, and jaundice. Characteristic histopathological lesions are lymphoid depletion with histiocytic replacement in lymphoid tissues as well as thymic atrophy (4, 6, 51, 110, 117).

Another clinical manifestation of PCVAD is PCV2-associated enteritis which can resemble chronic ileitis arising from an infection with the bacterium *Lawsonia intracellularis*. PCV2-assoicated enteritis affects 8 to 16 week-old piglets with clinical signs of diarrhea, unthriftiness, and enlarged mesenteric lymph nodes with a thickened intestinal mucosa (56). Three factors are considered in order to diagnose PCV2-associated enteritis: diarrhea, lesions found in Peyer patches but not in other lymph nodes, and PCV2 antigen or nucleic acid present within the lesions (61).

PCV2 can be involved in the porcine respiratory disease complex (PRDC). This PCVAD syndrome can occur when other pathogens, such as PRRSV, SIV, and *M. hyopneumonia*, are co-infecting along with PCV2. Clinical signs include a decreased growth rate, decreased feed efficiency, anorexia, fever, cough, and pneumonia in 8 to 26 week-old pigs (52, 60).

Although it is a rare form of PCVAD, PCV2-associated reproductive failure was first reported from Canada in 1999 (134) and typically affects new swine herds or gilt startups (86). Clinical manifestations include abortions, still births, fetal mummifications, and preweaning mortalities. Experimental intrauterine infection demonstrated that the heart is the primary site of

PCV2 replication in fetuses, and both early and late-term PCV2 infection can cause reproductive abnormalities (58, 115).

A highly fatal manifestation of PCVAD is porcine dermatitis and nephropathy syndrome (PDNS). PDNS was first described in 1993 in the United Kingdom (122), but wasn't found to be associated with PCV2 until 2000 (111). This syndrome typically affects grower pigs with signs of acute fever, lethargy, and raised purple skin lesions most prominent on the rear legs. Once clinical signs appear, death is common and can occur within three days of onset of clinical signs. PDNS develops by coinfection with other pathogens such as PRRSV (18, 126) and bacterial pathogens including *Pasteurella multocida*, *Haemophilus parasuis*, and *Bordetella bronchiseptica* (67, 127).

Diagnosis

There are three criteria for a definitive diagnosis of PCVAD within a herd: wasting, microscopic lesions of lymphoid depletion with histiocyte replacement, and the presence of PCV2 antigen or DNA in the lesions (117). Reduced T and B cell populations with lymphoid depletion are also characteristic of PCVAD. PCV2 infection decreases the expression of B-cell growth factor IL-4 and the cytotoxic T cell and macrophage-activating cytokine IL-2 (26) making PCV2 an immunosuppressive pathogen. It has also been shown that the degree of lymphoid depletion directly correlates with the amount of PCV2 antigen in tissues (27, 48, 116, 118). While lymphoid depletion is the hallmark pathological lesion of PCV2, the gold standard in detection of PCV2 antigen or nucleic acid is through polymerase chain reaction (PCR), *in situ* hybridization (ISH), and immunohistochemistry (IHC) (95).

Factors impacting PCVAD outcome

Even though a clinical manifestation typically involves co-infection with other pathogens, PCV2 is required for the development of PCVAD. Since not all pigs infected with PCV2 will develop a clinical disease, there are four main factors believed to impact the outcome of PCVAD: virus-dependent, host-dependent, co-infections, and immune modulation (95). There is very little difference between the genomic sequences (95.6-100% sequence homology) of PCV2 strains that cause severe disease in pigs and those that only result in a subclinical infection (49). It was shown that two amino acid changes in the capsid gene of the PCV2 genome significantly alter histopathological lesions in pigs, indicating that small changes can result in major pathological differences (37). There have also been reports that with the introduction of PCV2b into the United States, more severe PCVAD outbreaks have occurred than with those associated with PCV2a (17). There is also little difference in susceptibility of PCV2 infection between different breeds of pigs. Clinical PCAVD has been observed in both purebred and crossbred pigs (96). Co-infections, as mentioned previously, greatly impact the severity and outcome of PCV2 infection and are typically required to induce the full clinical spectrum of PCVAD. Finally, the effects of both immunostimulation and immunosuppression on disease caused by PCV2 have been studied. One study showed that immunostimulation with keyhole limpet hemocyanin in incomplete Freund adjuvant caused a more severe clinical disease in pigs when infected with PCV2 (64), which raises a concern about the effects of adjuvants in vaccines. As for immunosuppression, pigs infected with PCV2 after injection with cyclosporine had increased viral replication and a higher titer of virus compared to the controls, but did not develop clinical PCVAD (63).

Antibody response

Even though PCV2 is an immunosuppressive agent affecting B cells and decreasing lymphocyte proliferation, pigs infected with PCV2 seem to mount a strong PCV2-specific antibody response. Experimental PCV2 infection in colostrum-deprived pigs showed seroconversion between 14 and 28 dpi, and by 10 dpi pigs developed neutralizing antibodies that increased up to 21 dpi (5, 9, 64, 85, 106).

Treatment and prevention

There is no specific treatment once a pig has developed a clinical case of PCVAD. Treatment is usually supportive and depends upon the clinical signs and severity of the disease. It is important to identify all pathogens present in an animal since PCVAD usually involves co-infections and treatment can depend upon which pathogens are involved. Prevention and good management practices are the best ways to prevent PCV2 infection and PCVAD, especially in herd populations. Good management practices include minimizing stress, maintaining good hygiene and nutrition, preventing mixing of ages, and eliminating potential co-infecting agents. Supplementing feed with antibiotics to reduce secondary bacterial infections and vaccinating against *Mycoplasma* may also reduce the severity of PCV2 infection (92). Disinfecting buildings and transportation vehicles are recommended to reduce the chance of PCV2 spread and infection (113). A 20-point plan to control severe PCVAD once it has been introduced into a swine herd has been proposed (75). The main points of the plan include limiting pig to pig contact, reducing stress, and practicing good hygiene and nutrition. However, even on farms with strict management practices, PCV2 and PCVAD outbreaks still occur.

PCV2 vaccines

There are currently four vaccines available for PCV2 and the control of PCVAD; each based upon the PCV2a genotype. All are killed or recombinant subunit vaccines and have been successful in reducing mortality in piglets caused by PCV2 throughout North America, South America, and Europe (95). However, since PCV2b has become more prevalent worldwide and as viruses continue to evolve, it is unknown whether the current PCV2a-based vaccines can provide complete cross-protection against PCV2b and other emerging strains. Each of the four vaccines is described below:

1) Merial's CIRCOVAC® (Duluth, GA) is an inactivated PCV2 vaccine with an oil adjuvant for use in healthy female breeding-age pigs. The vaccine is available in Europe and Canada and has been successful in reducing PCV2 circulation and shedding in the first weeks of life of piglets born to vaccinated sows (13, 95).
2) Boehringer Ingelheim's Ingelvac CircoFLEX® vaccine (Ames, IA), available in the United States, is a capsid-based subunit vaccine expressed in an inactivated baculovirus. Decreased mortality from PCV2 infection in vaccinated versus unvaccinated pigs was demonstrated on four different Canadian finishing sites (28, 95).
3) Intervet Inc/Schering-Plough Animal Health (Kenilworth, NJ), also produces a capsid-based vaccine in a baculovirus which is suitable for pigs 3 weeks and older. The vaccine is marketed as Circumvent® PCV in the United States and Canada, while in Europe and Asia it is known as Porcilis® PCV. Studies including 35,000 pigs on 21 different farms showed that mortality of vaccinated pigs was reduced by 77.5% when compared with unvaccinated animals (46, 95).

4) One notable vaccine, the Pfizer Fort Dodge Suvaxyn® PCV2 One Dose™ (Fort Dodge and Wyeth Animal Health, Fort Dodge, IA), released in 2006 for pigs 4 weeks and older, is the first United States Department of Agriculture (USDA) approved and fully licensed PCV2 vaccine. The vaccine is an inactivated chimeric virus containing the replicase gene of non-pathogenic PCV1 with the capsid gene of pathogenic PCV2a (35-38). The genetically engineered vaccine has been shown to be attenuated in pigs and able to prevent viremia and lymphopenia associated with PCV2 morbidity (36, 38). A live version of the PCV1-2 chimera demonstrated to be genetically stable in vaccinated pigs (44) and could serve as a live vaccine candidate.

Molecular Breeding

In nature, viruses undergo genetic mutation and recombination, and through natural selection, those with increased fitness will continue to survive and replicate. This natural selection of viruses for desirable traits and improved physiological characteristics can take many years and multiple generations to achieve. Molecular breeding through DNA shuffling accelerates the natural evolutionary process within the laboratory using novel *in vitro* techniques. By using a group of related parental genes, DNA shuffling can produce thousands of recombinants which can be screened and selected for improved and desirable characteristics (23, 73). DNA shuffling can incorporate many parental alleles, versus only two in natural reproduction. There are two primary techniques for molecular breeding of viruses: multi-gene shuffling and synthetic DNA shuffling.

The traditional multi-gene DNA shuffling method involves digesting a pool of related parental genes with DNase I and allowing the fragments to randomly reassemble using PCR

technology (24, 123, 138) (Figure 1.2). The fragments generated from DNase I digestion range between 5-10% of the original gene size, and these fragments can reassemble in many configurations leading to thousands of different shuffled gene sequences. With this method, there is little control over how the DNA fragments will recombine, and a high degree of homology between the parental genes is necessary for successful and complete recombination.

Synthetic DNA shuffling involves the use of synthetic degenerate oligonucleotide primers in which every amino acid from a set of parents is allowed to recombine independently of every other amino acid (89) (Figure 1.3). With synthetic DNA shuffling, starting parental genes are unnecessary and specific mutations can be incorporated. With the development of bioinformatics, statistical, and sequence analysis programs, it is possible to use a vast pool of parental genes to capture additional diversity, select for specific domains, and shuffle genes with low homology. Therefore, variations of shuffled products can be generated that would not have been created through the fragmentation-based shuffling method.

Regardless of the technique, the key feature of molecular breeding in vaccine development is that the resulting shuffled viruses can be selected for improved cross-reactivity and enhanced immunogenicity against genetically different strains of a pathogen (73, 136). Other applications of DNA shuffling include improving protein and viral expression, generating viruses with improved replication kinetics and altered tropism, developing vaccines for infectious diseases and allergies, increasing stability and biological activity of specific genes, and generating antigens with improved affinity to antibodies (74).

A library of shuffled products may potentially contain millions of different chimeras, up to 10^9 individual clones (73), but only a small portion of these shuffled genes may be biologically active or contain the wanted characteristics. Therefore, an effective screening

process must be available in order to select for the desired product. In a fragmentation-based shuffling study, multiprobe DNA microarrays were used to analyze the proportion of chimeric genes after *in vitro* recombination. Fluorescent oligonucleotides were used to sequence map and assess recombination biases (1). In another study, probe hybridization in a gene chip format was used to determine parental gene bias and identify where specific crossovers took place (57). With synthetic DNA shuffling, an effective screening strategy can be achieved with the addition of an antibody tag to identify full length constructs and sort bright-expressing clones with a fluorescence-activated cell sorter (74). Bioinformatics have greatly facilitated and accelerated the identification of lead chimeric vaccine candidates when used with display technologies and high through-put screening.

There are many examples in which DNA shuffling strategies have been employed to develop novel vaccine candidates. Yang *et al.* (2002), recombined the envelope genes of the four Dengue viruses (DEN-1-4) into a single chimeric gene capable of producing neutralizing antibodies against all four Dengue serotypes in mice. Sera from the mice showed improved cross reactivity when measured by enzyme linked immunosorbant assay (ELISA) and virus neutralization assays (137). Similarly, shuffling of the E1 and E2 genes of three equine encephalitis alphaviruses (Venezuelan, western, and eastern equine encephalitis virus) demonstrated that the resulting chimeras induced cross-reactive antibody responses with five distinct encephalitic alphavirus isolates (101). A final example of molecular breeding was the shuffling of the human hepatitis B virus surface antigen gene with homologous genes from woodchuck, woolly monkey, and chimpanzee/gibbon hepatitis B viruses. The chimeras produced were more immunogenic than the commercially available hepatitis B virus vaccine when in both DNA and protein immunization form (135). These are just a few out of many

examples in which DNA shuffling and screening strategies have been employed to improve vaccine candidates for infectious diseases.

Figure 1.1: Genomic organization of PCV1 and PCV2.

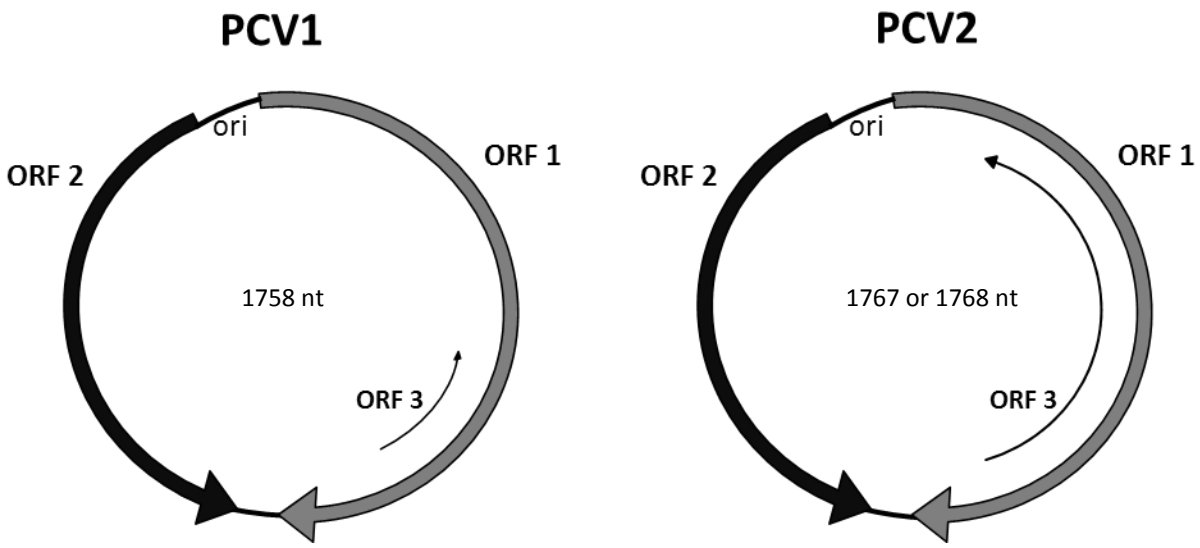
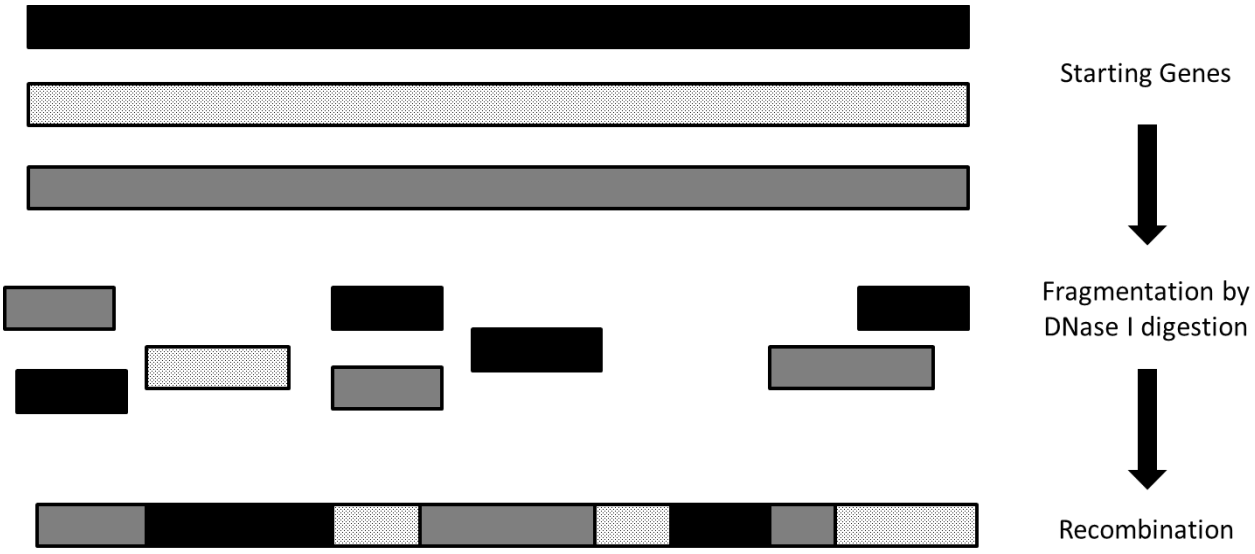
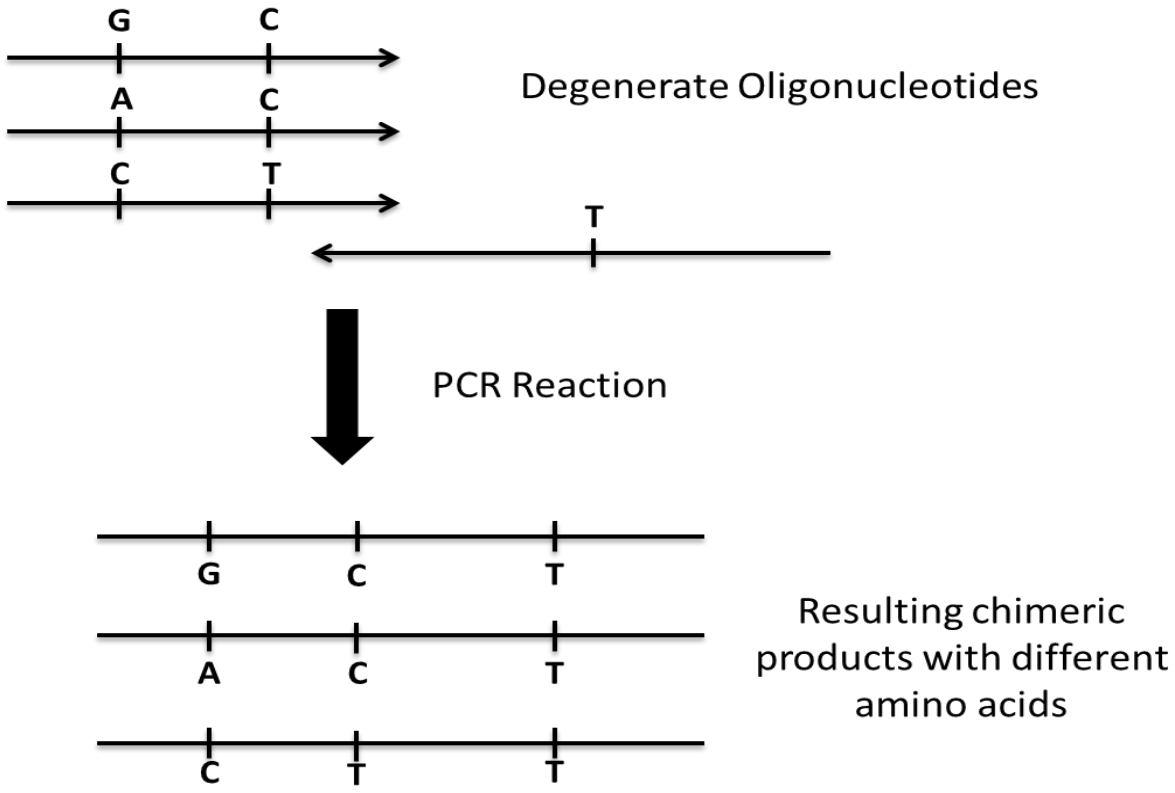


Figure 1.2: Traditional multi-gene DNA shuffling method.



Result – shuffled chimeric viruses with representation from each parental gene

Figure 1.3: Synthetic DNA shuffling method with use of short degenerate oligonucleotide primers.



Chapter 2

Molecular Breeding of Porcine Circovirus Type 2 by Synthetic DNA Shuffling

Introduction

Porcine circovirus (PCV) was first discovered in 1974 as a contaminant of the porcine kidney PK-15 cell line. It is a non-enveloped, single-stranded, DNA virus belonging to the family *Circoviridae* (3, 4, 95, 128). PCV, approximately 1.7kb in length, has a simple genomic organization consisting of two main open reading frames (ORF): ORF1 (942 bp) encodes two replicase proteins, and ORF2 (702 bp) encodes the immunogenic capsid protein (14, 50). There are currently two distinct types of PCV which share similar genomic organizations and approximately 68-75% nucleotide sequence identity (16, 34). These two types are designated as PCV type 1 (PCV1) and PCV type 2 (PCV2) (5). PCV1 is considered to be non-pathogenic in swine (6, 8) while PCV2, discovered in Canada in 1997, is associated with a clinical disease in piglets known as postweaning multisystemic wasting syndrome (PMWS) (3, 32, 51, 70, 88).

In 2006, the American Association of Swine Veterinarians (AASV) approved a name change of PMWS to porcine circovirus-associated disease (PCVAD) in order to include all clinical manifestations arising from infection with PCV2 (95). These clinical manifestations include wasting, jaundice, weight loss, enlarged lymph nodes, lymphoid depletion, and respiratory problems in 8 to 15 week-old piglets (19, 32, 51). PCV2 has also been associated with more serious complications including pneumonia, nephritis, enteritis, dermatitis, reproductive failure, and death (52, 56, 60, 66, 90). PCV2 and PCVAD have been reported worldwide with prevalence rates up to 80% in swine herds from some affected countries. Locations where PCVAD has been reported include, but are not limited to, the United States,

Europe, China, Canada, and Australia (7, 31, 40, 59). PCV2 has become one of the most economically important viral pathogens affecting swine populations worldwide.

There are three distinct genotypes of PCV2 documented to date: PCV2a, PCV2b, and PCV2c. Prior to 2005, the predominant circulating genotype throughout North America was PCV2a, while PCV2b isolates were found primarily in Europe and China (17, 34). Over the past six years, PCV2b has become more prevalent in the United States and Canada and has been associated with more severe PCVAD cases (43). The third genotype, PCV2c, was isolated from Denmark in the 1980's, but only from non-diseased herds (31).

Four PCV2 vaccines are commercially available for the control of PCV2 and PCVAD. These vaccines are either killed or recombinant vaccines based upon the PCV2a genotype (35, 38, 95). Since there is up to a 10% nucleotide sequence difference between the three PCV2 genotypes, and since PCV2b has become the predominant circulating genotype (31, 69, 91), it is unknown if the current vaccines can provide complete cross-protection against infection with PCV2b. Therefore, a broadly-protective candidate vaccine is desirable which can take into consideration the genetic heterogeneity of PCV2 as well as potential mutations that arise through vaccine selection pressure.

In vitro techniques known as molecular breeding, or DNA shuffling, can be used to create chimeric viruses from a pool of related parental viruses and facilitate the development of potential candidates for broadly-protective vaccines. Past studies have used molecular breeding to produce shuffled gene sequences which are then selected for improved cross-reactivity and enhanced functional properties (74, 135, 136). There are two common techniques used to generate shuffled viruses: multi-gene DNA shuffling and synthetic DNA shuffling. The multi-gene method involves fragmenting a pool of related parental genes and allowing the fragments to

randomly reassemble using PCR technology. Synthetic DNA shuffling uses short synthetic degenerate oligonucleotide primers in which every amino acid variation from a set of parental gene sequences is allowed to be incorporated independently of every other amino acid. In a study conducted by Ness *et al*. (2002), it was demonstrated that synthetic DNA shuffling produced chimeras with a higher degree of amino acid diversity when compared to the multi-gene shuffling method. Synthetic oligonucleotides for production of a shuffled library can be constructed directly from sequence information available in GenBank database. With synthetic DNA shuffling, specific mutations can be incorporated and starting parental genes are unnecessary. Regardless of the chosen methodology, the key feature of molecular breeding in vaccine development is the resulting shuffled products can be selected for improved cross-reactivity and enhanced immunogenicity against multiple strains of a pathogen.

With the use of synthetic DNA shuffling, we report on the generation of shuffled PCV2 capsid genes encompassing genetic heterogeneity from all available PCV2a, PCV2b, and PCV2c strains. Viable chimeric PCV2 viruses containing a shuffled capsid were also identified and isolated *in vitro*.

Materials and Methods

Designing degenerate oligonucleotide primers spanning the ORF2 of PCV2

All available PCV2a, PCV2b, and PCV2c capsid gene sequences from GenBank database were retrieved, aligned, and compared using BioEdit Sequence Alignment Editor software (Hall, T.A., 1999). Amino acid positions with greater than 1% sequence variation among all 853 PCV2 strains were selected as positions to be included in the shuffling. A total of seventy-three oligonucleotide primers were synthesized to incorporate appropriate degeneracies within the

PCV2a (Invitrogen Corporation, Life Technologies, Carlsbad, CA) (Figure 2.1). Several oligonucleotide primers for certain stretches of DNA were synthesized in order to include diversity unable to be expressed by a single degenerate primer. The degenerate primers used in this study are listed in Table 2.1.

Generation of shuffled chimeric PCV2 capsid sequences by PCR technology

Oligonucleotide primers were paired and annealed together to create nine double-stranded DNA fragments using a PCR annealing reaction with PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies, Santa Clara, CA) (Figure 2.2a). Reactions took place in 25 µl total volume with primer pairs at 1µM final concentration each. The annealing PCR reaction consisted of one cycle of initial denaturation at 95 ºC for 3 min, 20 cycles of denaturation at 95 ºC for 30 sec, annealing at 54 ºC for 30 sec, and extension at 72 ºC for 20 sec, followed by a final extension at 72 ºC for 8 min. Each DNA fragment was separated by electrophoresis on a 3% agarose gel, excised, and gel purified using QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD). The resulting nine DNA fragments were then paired and fused together through several rounds of overlap extension PCR (Figure 2.2a) using PfuUltra II Fusion HS DNA Polymerase. Reactions took place in 50 µl total volume with outer primers at a final concentration of 200 nM and 100-200 ng of total template DNA. The overlap extension PCR reaction consisted of one cycle of initial denaturation at 95 ºC for 3 min, 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 60 ºC for 30 sec, and extension at 72 ºC for 1 min, followed by a final extension at 72 ºC for 8 min. Each fragment was separated by electrophoresis on a 2% agarose gel and the expected band was excised and gel-purified between each round of overlap extension PCR. The final purified shuffled PCR capsid products were TA

cloned into pCR2.1 (Invitrogen Corp., Carlsbad, CA) according to manufacturer's instructions and sequenced (Virginia Bioinformatics Institute, Blacksburg, VA). The sequences were aligned and compared using BioEdit Sequence Alignment Editor to confirm the presence of properly shuffled products with genetic representation from PCV2a, PCV2b, and PCV2c. Individual capsid sequences that displayed proper shuffling were selected to be inserted into the backbone of the PCV2a infectious DNA clone. The library of shuffled PCR capsid products, potentially containing millions of different shuffled clones, was also cloned into the backbone of the PCV2a infectious DNA clone.

Insertion of shuffled chimeric PCV2 capsid genes into the PCV2a infectious DNA backbone clone

To create full-length shuffled chimeric PCV2 viruses (PCV2-S), the synthetically shuffled PCR capsid gene products were inserted into a PCV2a infectious DNA clone (Figure 2.2b). The PCV2a isolate 40895 replicase gene and origin of replication were synthesized by GenScript (Piscataway, NJ) and cloned into pBluescript II SK (+) (PCV2a-pBSK). Synthetically shuffled PCR capsid products were digested using MscI and BsmI restriction sites flanking the ORF2 gene and then cloned into PCV2a-pBSK. Clones were sequenced to confirm the proper insertion. Prior to transfection, the PCV2-S genomes were digested out of pBSK II using SacII restriction endonuclease and self-ligated using T4 DNA ligase (Invitrogen Corp., Carlsbad, CA) to create circular PCV2-S infectious DNA clones.

Production of chimeric PCV2-S infectious virus stock

To produce a stock of chimeric PCV2-S infectious viruses, PK-15 cells were transfected with circularized PCV2-S DNA clones. PK-15 cells were maintained at 37 ºC in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2 X antibiotic-antimycotic. PK-15 cells were grown in a T-25 flask until they reached approximately 50% confluency. The cells were transfected with 600 ng concatomerized PCV2-S ligation mixture (600 ng each individual PCV2-S clone and 600 ng PCV2-S library) using lipofectamine LTX (Invitrogen Corp., Carlsbad, CA) and incubated at 37 ºC for 6 hr. Following incubation, 6 ml of growth media was added to the inoculum. After incubation at 37 ºC for 3 days, cells were trypsinized and concentrated into 1 ml MEM without FBS or antibiotics. Cells were frozen at -80 ºC and thawed 3 times to release the virus and generate PCV2-S virus stocks.

To determine the replication-competency of chimeric PCV2-S genomes, transfected PK-15 cells were grown to 100% confluency in a 48-well plate and stained by immunofluorescence assay (IFA) with a PCV2-specific antibody. Cells were fixed by removing growth media and adding 300 µl of 80% acetone to each well and incubating at 4 ºC for 15 min. Cells were washed once with phosphate buffered saline (PBS), and 75 µl of swine antiserum containing anti-PCV2 rep antibodies (a generous gift of Dr. Andrew Cheung, National Animal Disease Center, Ames, IA) at 1:250 dilution was added. The cells were then incubated at 37 ºC for 45 min. Following incubation, cells were washed 3 times with PBS to remove the primary antibody, and 75 µl of fluorescein isothiocyanate (FITC)-labeled anti-swine antibody at 1:50 dilution was added to each well and incubated again at 37 ºC for 45 min. A final wash with PBS removed the secondary antibody, and cells were covered with fluoromount and visualized under a fluorescent microscope.

Determination of in vitro infectivity and viability of chimeric PCV2-S viruses

The infectivity and *in vitro* viability of PCV2-S viruses was determined by inoculating new PK-15 cells grown to approximately 50% confluency in a 48-well plate. Cells were infected with 100 µl of undiluted and 100 µl 1:10 diluted individual PCV2-S and library PCV2-S virus stocks. After incubation for 1 hr at 37 ºC, 400 µl growth media was added to each well and cells were incubated at 37 ºC for 3 days to reach 100% confluency. *In vitro* viability of PCV2-S viruses was determined using IFA with an anti-PCV2 Rep specific antibody as described above.

Sequence confirmation of individual chimeric PCV2-S viruses

DNA from PK-15 cells infected with individual PCV2-S viruses was extracted using QIAamp DNA Mini and Blood Mini Kit (QIAGEN Sciences, Germantown, MD). The shuffled capsid DNA was PCR amplified using Platinum PCR Supermix High Fidelity Polymerase (Invitrogen Corp., Carlsbad, CA) in 50 µl reactions with outer capsid primers fA and BsmI (Table 2.1) at a final concentration of 200 nM. The PCR reaction consisted of one cycle of initial denaturation at 95 ºC for 3 min, 35 cycles of denaturation at 95 ºC for 30 sec, annealing at 54 ºC for 30 sec, and extension at 68 ºC for 1 min, followed by a final extension at 68 ºC for 8 min. The resulting PCR products were separated by electrophoresis on 1.5% agarose gel and gel-purified. Recovered PCR products were sequenced with primer fA to confirm the recovery of chimeric PCV2-S virus.

Isolation of viable chimeric infectious clones from the PCV2-S library

DNA from PK-15 cells infected with the PCV2-S library was extracted and PCR amplified using outer capsid primers fA and BsmI as described above. Following gel-extraction

and purification, the amplified capsid product was TA cloned into pCR2.1 and transformed into competent *E. coli*. Individual colonies obtained from the transformation were sequenced. These clones were inserted back into PCV2a-pBSK plasmid, then transfected into PK-15 cells. Detection of viable infectious virus was performed by IFA as described above.

Addition of a hemagglutinin (HA) tag to the C-terminal end of PCV2 ORF2

In order to facilitate the selection and screening process of PCV2-S viruses, an influenza virus hemagglutinin (HA) tag (YPYDVPDYA) was inserted into the carboxy (C') terminus of the capsid gene (Figure 2.6a). It has been previously shown that addition of the 9 amino acid HA tag at the C' end of the PCV2 capsid does not alter viability of the virus (10). The HA sequence was inserted into the shuffled capsid by overlap extension PCR (primers listed in Table 2.1) and cloned into PCV2a-pBSK using reaction conditions as described above. Infectious HA tagged PCV2-S (PCV2-S-HA) virus stocks were produced and used to infect PK-15 cells as described above. PCV2-S-HA virus was visualized by IFA as described above except using a FITC-labeled anti-HA tag monoclonal antibody (MAb; Sigma, St. Louis, MO).

Results

Bioinformatics analysis of PCV2 ORF2 sequences

In the fall of 2009, all PCV2 capsid gene sequences available in GenBank database (n=853) were retrieved and aligned using BioEdit. The sequences were compared to identify amino acid positions with the greatest diversity among all capsid gene sequences. A total of fifty-five amino acid positions (55/233; 23.6%) were identified to have variation in greater than 1% of the 853 strains or contained specific PCV2c amino acids (Table 2.2). Amino acid

positions specific to PCV2c were introduced since only 3 PCV2c sequences were available in GenBank database (3/853; 0.35%). Each of these 55 positions had 2-6 different possible amino acids. A total of 73 degenerate oligonucleotide primers (47-78 nt in length) were synthesized to represent the genetic diversity of all PCV2 capsid sequences. Several oligonucleotide primers for specific stretches of DNA were synthesized in order to include diversity that were unable to be expressed by a single degenerate primer.

Chimeric PCV2 capsid gene sequences containing genetic diversity from PCV2a, PCV2b, and PCV2c were generated using synthetic DNA shuffling

Using PCR, full-length chimeric PCV2 capsid gene sequences were successfully generated from the degenerate oligonucleotide primers. Nine sets of oligonucleotide primers were grouped and annealed together to create nine double stranded DNA fragments (87-111 bp in length). Several consecutive overlap extension PCR reactions were used to fuse these DNA fragments together resulting in a full-length chimeric PCV2 capsid gene. The capsid gene was successfully cloned into pCR2.1, and a library was created after transforming competent *E. coli*. 100 different clones were isolated from the library and sequenced. Sequence analysis revealed that 90 of the clones contained unwanted point and frame shift mutations, while 10 of the clones were properly shuffled with genetic representation from all the PCV2 genotypes (PCV2a, PCV2b, and PCV2c). The 10 properly shuffled individual clones and the capsid library were inserted into PCV2a-pBSK to create full-length chimeric PCV2-S DNA genomes.

Transfection of PK-15 cells demonstrated replication-competency of the full-length chimeric PCV2-S genomes

To determine the replication-competency of the PCV2-S genomes, concatomerized PCV2-S DNA was transfected into PK-15 cells. On day 3 post-transfection, cells transfected with a wildtype PCV2a genome resulted in strong nuclear staining by IFA (Figure 2.3a), while mock transfection of PK-15 cells yielded no nuclear signal (Figure 2.3b). Three individual chimeric PCV2-S genomes (clone IDs: 16-3, 16-4, and 16-7) displayed clear nuclear fluorescence (Figure 2.3c) at 3 days post-transfection indicating the viability of these 3 clones. The other seven individual PCV2-S genomes showed no nuclear fluorescence following transfection indicative of nonviable clones. Fluorescence signal was also detected in cells transfected with the PCV2-S library genome (Figure 2.3d) indicating that chimeric PCV2-S genomes are replication-competent in PK-15 cells.

Infection of PK-15 cells demonstrated viable infectious chimeric PCV2-S viruses

Cell lysates from transfected cells were used to infect new PK-15 cells to test for *in vitro* viability and infectivity of the chimeric viruses. Nuclear fluorescence signal was detected in cells infected with wildtype PCV2a (Figure 2.4a) at 3 days post-infection (dpi), while mock infection showed no fluorescent signal (Figure 2.4b). Nuclear fluorescence was also detected in PK-15 cells infected with three individual chimeric PCV2-S viruses (16-3, 16-4, and 16-7) (Figure 2.4c), as well as with the PCV2-S library (Figure 2.4d) on 3 dpi. These results indicate that chimeric PCV2-S viruses are viable and infectious in PK-15 cells. Following infection, sequence analysis confirmed that cells were indeed infected by the appropriate individual PCV2-S viruses and no additional mutations arose during *in vitro* replication (Figure 2.5). The three

individual chimeric PCV2-S viruses contained genetic representation from PCV2a, PCV2b, and PCV2c, and each was shuffled differently from one another.

Individual clones isolated from the PCV2-S library were not viable in PK-15 cells

Five shuffled capsid gene sequences containing no unwanted point or frame shift mutations were successfully isolated from the PCV2-S library following infection of PK-15 cells. Each capsid was individually cloned back into PCV2a-pBSK and subsequently transfected into PK-15 cells. PCV2-specific nuclear fluorescence was not detected from any of the isolates by IFA following transfection. Infecting PK-15 cells with the individual library isolates also resulted in no fluorescent signal indicating that these individual viruses isolated from the PCV2-S library were not replication-competent or viable *in vitro*.

Addition of an HA tag resulted in the recovery viable PCV2-S-HA viruses

An HA tag was successfully inserted into the C-terminal end of the shuffled capsid to facilitate the screening process of viable chimeric PCV2-S viruses using an anti-HA tag monoclonal antibody. On day 3 post-transfection, nuclear fluorescence was detected in PK-15 cells transfected with PCV2-S-HA library DNA (Figure 2.6b), indicating that addition of an HA tag continued to yield replication-competent PCV2-S genomes. PCV2-specific nuclear fluorescence signal was also detected at 3 dpi in PK-15 cells infected with PCV2-S-HA library (Figure 2.6c) demonstrating that HA-tagged PCV2-S viruses are viable and infectious *in vitro*.

Discussion

PCVAD causes severe economic losses worldwide in the swine industry with clinical manifestations ranging from a subclinical infection to serious complications including progressive weight loss, pneumonia, enteritis, reproductive failure, and high mortality in pigs (4, 32, 54, 95, 117). Prior to 2005, PCV2a was the only genotype present in North America, while PCV2b was predominantly found in Europe and China (17, 34). However, over the past six years, PCV2b has become the main circulating genotype and has been linked to more severe PCVAD outbreaks in certain areas of the United States and Canada (43). Even though the available PCV2 vaccines have been shown to be effective throughout North America, South America, and Europe (55, 95, 97, 117), each vaccine is based upon the PCV2a genotype. Studies have tested the effectiveness of the PCV2a-based vaccines against PCV2b challenge, but results did not demonstrate 100% cross-protection between the two genotypes (41, 42, 98). A challenge study using a PCV2a capsid-based subunit vaccine in a baculovirus system (Porcilis® PCV, Intervet International, The Netherlands) demonstrated that the vaccine was able to prevent viremia in all vaccinated animals regardless of the genotype, but nasal and fecal shedding was not fully prevented in pigs challenged with PCV2b (41). Therefore, a novel vaccine is needed that can take into consideration the heterogeneity of PCV2 and provide equal cross-protection against all three PCV2 genotypes.

In this study, synthetic DNA shuffling was used to generate shuffled chimeric PCV2 capsid gene sequences from 73 synthetic degenerate oligonucleotide primers engineered to include genetic diversity from capsid gene sequences of 853 published PCV2a, PCV2b, and PCV2c strains. Viable full-length chimeric PCV2 viruses with a shuffled capsid were successfully generated by DNA shuffling. Other studies have used the techniques of molecular

breeding to obtain shuffled gene sequences with enhanced functional properties, but all from smaller pools of parental genes. Using the traditional multi-gene shuffling method, Soong *et al*. (2000) shuffled the envelope genes of six murine leukemia viruses (MLV) and identified a chimeric virus that had completely new tropism for a different cell line. In another multi-gene shuffling study, nine capsid genes of adeno-associated virus (AAV) were shuffled, and a single chimeric clone was isolated from a cell line previously shown to have low permissiveness to AAV (71). In a study using synthetic shuffling, diversity from 15 subtilisin genes encoded in 30 degenerate oligonucleotide primers resulted in active enzymes containing amino acid combinations that were not obtained through the traditional shuffling method (89).

The overlap extension PCR procedure was effective in producing shuffled capsid gene sequences, but sequence analysis revealed that the majority of the PCR capsid products contained unwanted point and frame shift mutations. These mutations were most likely due to lack of purification after synthesis of the degenerate primers. The purification level used in this study was desalted, versus more expensive polyacrylamide gel electrophoresis (PAGE) primers. Desalted primers are processed through a chromatography column which removes salts but not faulty sequences, while PAGE purified primers have erroneous oligonucleotides removed from the correct products. By using lower primer purification, there was a higher probability that faulty primers were present in the PCR reactions. Other studies in our lab have shown that use of PAGE purified primers resulted in fewer unwanted mutations. Another factor responsible for the unwanted mutations may be caused by the DNA polymerase and consecutive rounds of overlap extension PCR used to generate the shuffled chimeric capsid. DNA polymerases with exonuclease activity such as Pfu are used to reduce the introduction of amplification errors in

PCR products, but high fidelity polymerases can still incorporate errors (Pfu error rate - 1.3×10^{-6} mutation frequency/bp/duplication) (20).

We were successful in generating and identifying live chimeric PCV2 viruses containing a shuffled capsid. Three of the ten PCV2-S viruses tested for *in vitro* viability displayed PCV2-specific nuclear fluorescence by IFA following infection of PK-15 cells indicating viable and infectious shuffled chimeric viruses. The seven nonviable clones likely contained amino acid combinations that resulted in deleterious or lethal phenotypes. Changing the amino acid composition of viral proteins can disrupt structure, function, and formation of infectious virus particles. Fenaux et al. (2004) demonstrated that two amino acid changes in the PCV2 capsid increased viral replication *in vitro* but attenuated the PCV2 virus *in vivo* (37). Similarly, mutating three amino acids in the capsid protein of a hepatitis E virus also resulted in decreased *in vivo* viral replication (21). In a study conducted by Cortazzo *et al*. (2002), codon substitutions in a specific turn between two alpha helixes of a fatty acid binding protein resulted in protein misfolding, decreased solubility, and induction of stress response elements when expressed in *E. coli* (22). Even mutating a single amino acid can result in deleterious phenotypes. It was demonstrated that replacing a specific amino acid in a measles virus fusion glycoprotein with those containing a polar or charged side chain completely inhibited or greatly reduced protein expression (105).

Because the capsid gene was shuffled randomly, it was impossible to use the standard anti-PCV2 capsid monoclonal antibody for detection of PCV2 in the screening process. Instead, a polyclonal serum containing both anti-capsid and anti-replicase antibodies was used, resulting in a bright nuclear fluorescence from the PCV2a wildtype and decreased fluorescent signal intensity from the shuffled viruses. The replicase is not the immunogenic protein of PCV2 and

has lower expression level compared to the capsid, resulting in less antibody binding and a decreased fluorescent signal intensity. In order to allow for staining of the shuffled capsids, an influenza virus HA tag was added to the C' terminus of the shuffled capsid and detected by use of an anti-HA tag antibody. The PCV2-S-HA library displayed specific nuclear fluorescence following both transfection and infection in PK-15 cells indicating that the HA tag does not inhibit *in vitro* replication-competency or viability, consistent with a previous study in which an HA tag was fused in frame to the C' terminus of the PCV2 capsid (10). However, separation of these viable viral genomes from the rest of the library was unsuccessful due to difficulties in virus isolation, as there are no plaque assay systems for PCV.

As viable chimeric PCV2-S viruses were identified *in vitro,* future studies on *in vivo* viability and infectivity are warranted. An animal study is required in order to determine whether the chimeric PCV2-S viruses can infect and replicate to a high enough titer for a pig to mount an immune response. Serum collected from pigs can then be tested for a PCV2 capsid specific antibody response, specifically one that produces antibodies capable of neutralizing each PCV2 genotype. Pathogenicity of the chimeric PCV2-S viruses will be determined through examination of histopathological lesions, viremia and serum viral loads, lymphoid tissue viral loads, and PCV2 specific antigen present in tissues following infection with the PCV2-S viruses.

In conclusion, results from this thesis study demonstrate that synthetic DNA shuffling is an effective method to produce shuffled PCV2 capsid gene sequences with genetic representation from PCV2a, PCV2b, and PCV2c. Our results also show that chimeric PCV2 viruses containing a shuffled capsid are viable and infectious *in vitro,* and addition of an HA tag at the C' end of the shuffled capsid does not hinder the viability of the chimeric viruses in PK-15 cells. Further

study is needed to characterize the *in vivo* viability and pathogenicity of the chimeric PCV2-S viruses.

Figure 2.1: Oligonucleotide primers designed for synthetic DNA shuffling of PCV2 ORF2. Diversity of 853 PCV2 capsid gene sequences from PCV2a, PCV2b, and PCV2c was encoded in a total of 73 synthetic degenerate oligonucleotide primers. Relative amino acid positions in which variation was incorporated are indicated by an X. Several primers were needed in certain areas to encode diversity that was unable to be expressed by a single oligonucleotide primer.

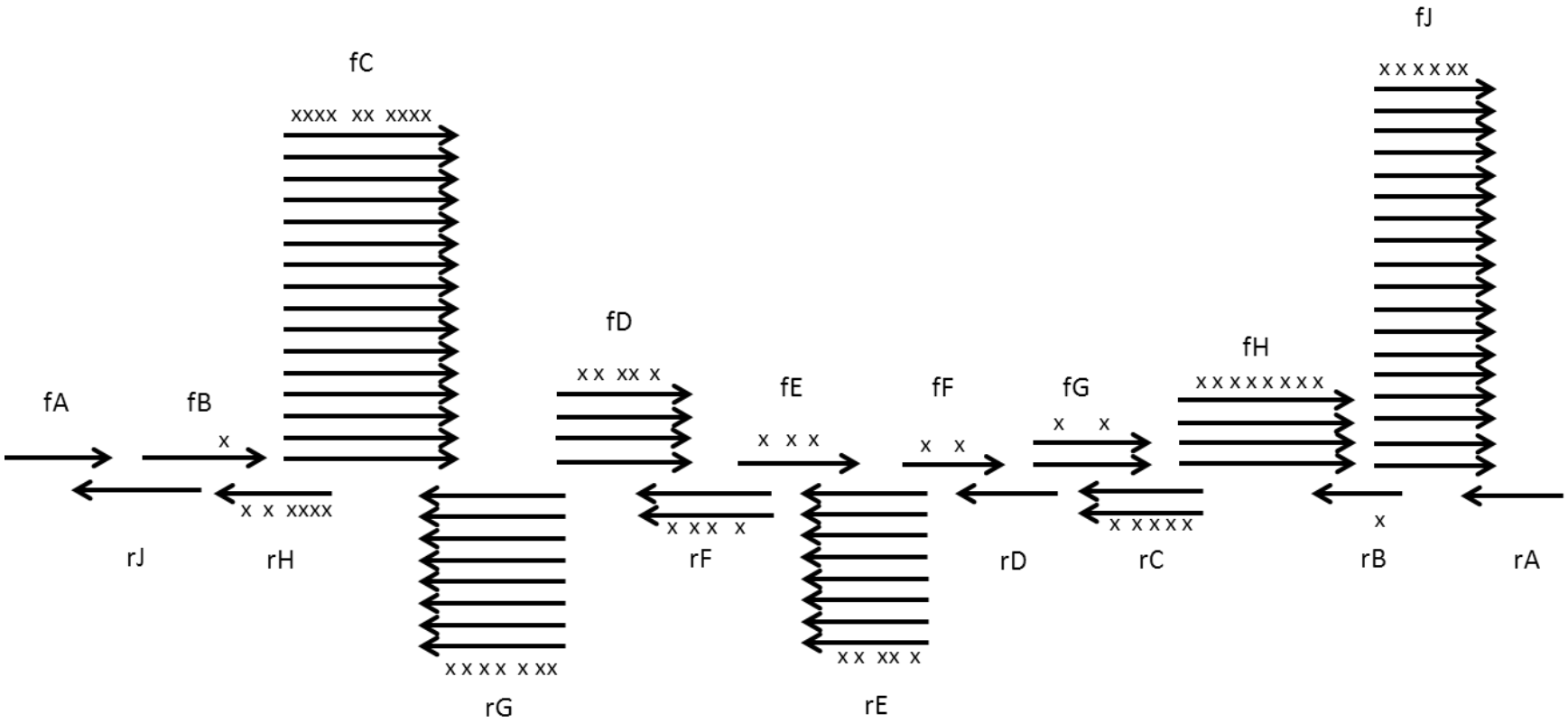
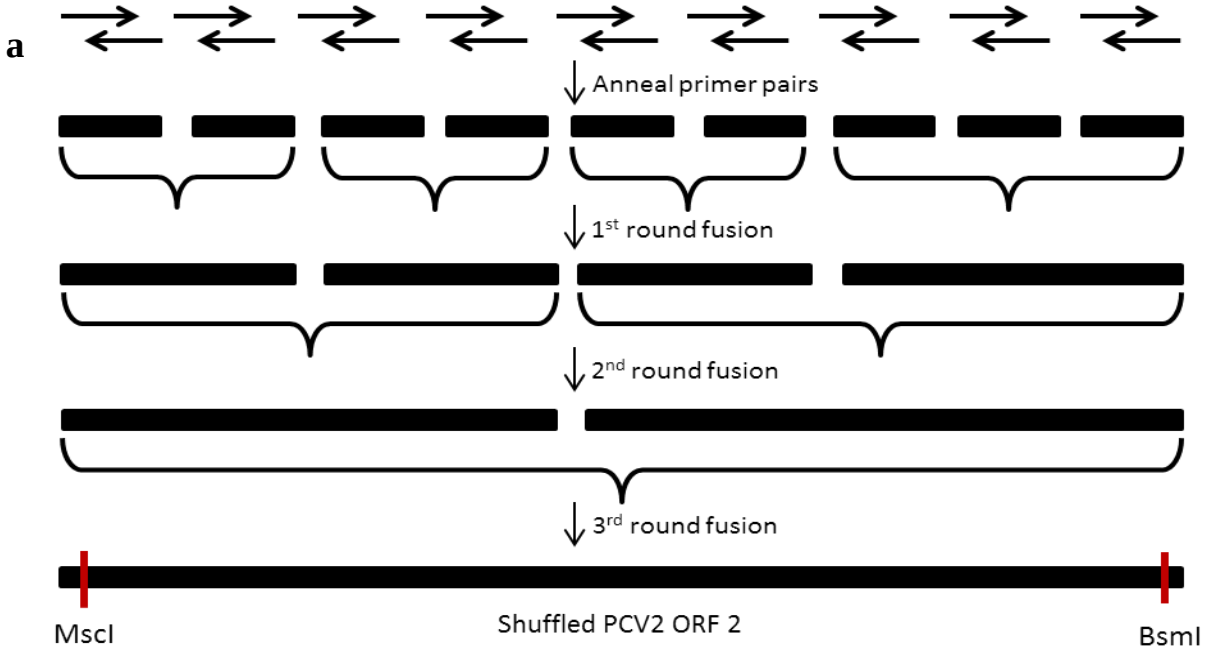


Figure 2.2: Construction of chimeric PCV2-S library. (a) Stepwise configuration of the annealing PCR and overlap extension PCR reactions to create shuffled PCV2 capsid gene sequences. (b) Insertion of the shuffled capsid gene into PCV2a-pBSK to create chimeric PCV2-S viruses.



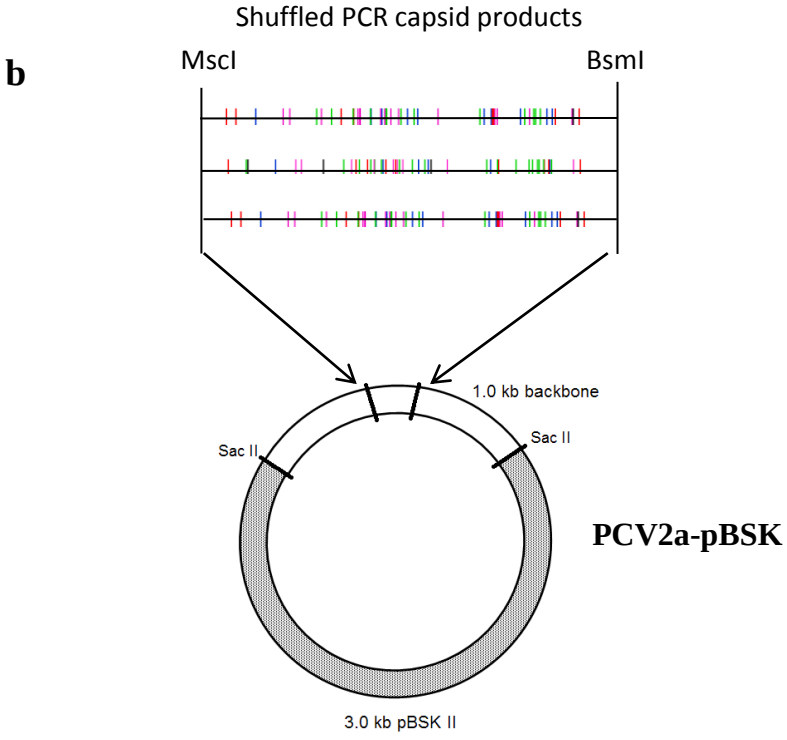


Figure 2.3: IFA pictures of PK-15 cells transfected with chimeric PCV2-S DNA using swine antiserum containing anti-PCV2 rep antibodies. (a) Nuclear staining of PK-15 cells transfected with PCV2a wild type positive control DNA clone (100x magnification); (b) Negative control, mock infection (100x); (c) Nuclear staining of PK-15 cells transfected with three identified individual PCV2-S clones (16-3, 16-4, and 16-7, all 200x); (d) Nuclear staining of PK-15 cells transfected with the PCV2-S library DNA mixture (200x).

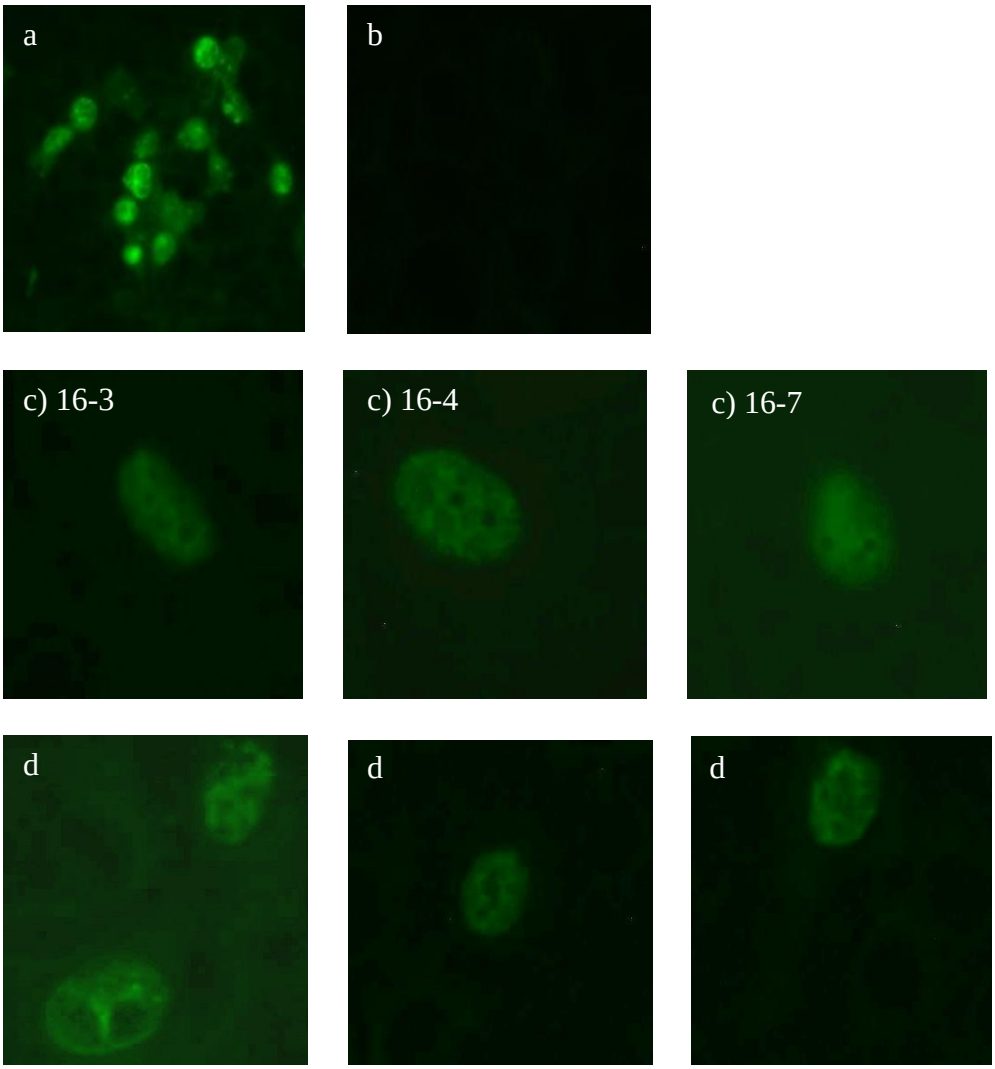


Figure 2.4: IFA pictures of PK-15 cells infected with chimeric PCV2-S viruses using swine antiserum containing anti-PCV2 rep antibodies. (a) Nuclear staining of PK-15 cells infected with PCV2a wild type positive control virus; (b) Negative control, mock infection; (c) Nuclear staining of PK-15 cells infected with three identified individual PCV2-S viruses (16-3, 16-4, and 16-7); (d) Nuclear staining of PK-15 cells infected with the PCV2-S library. All pictures at 200x magnification.

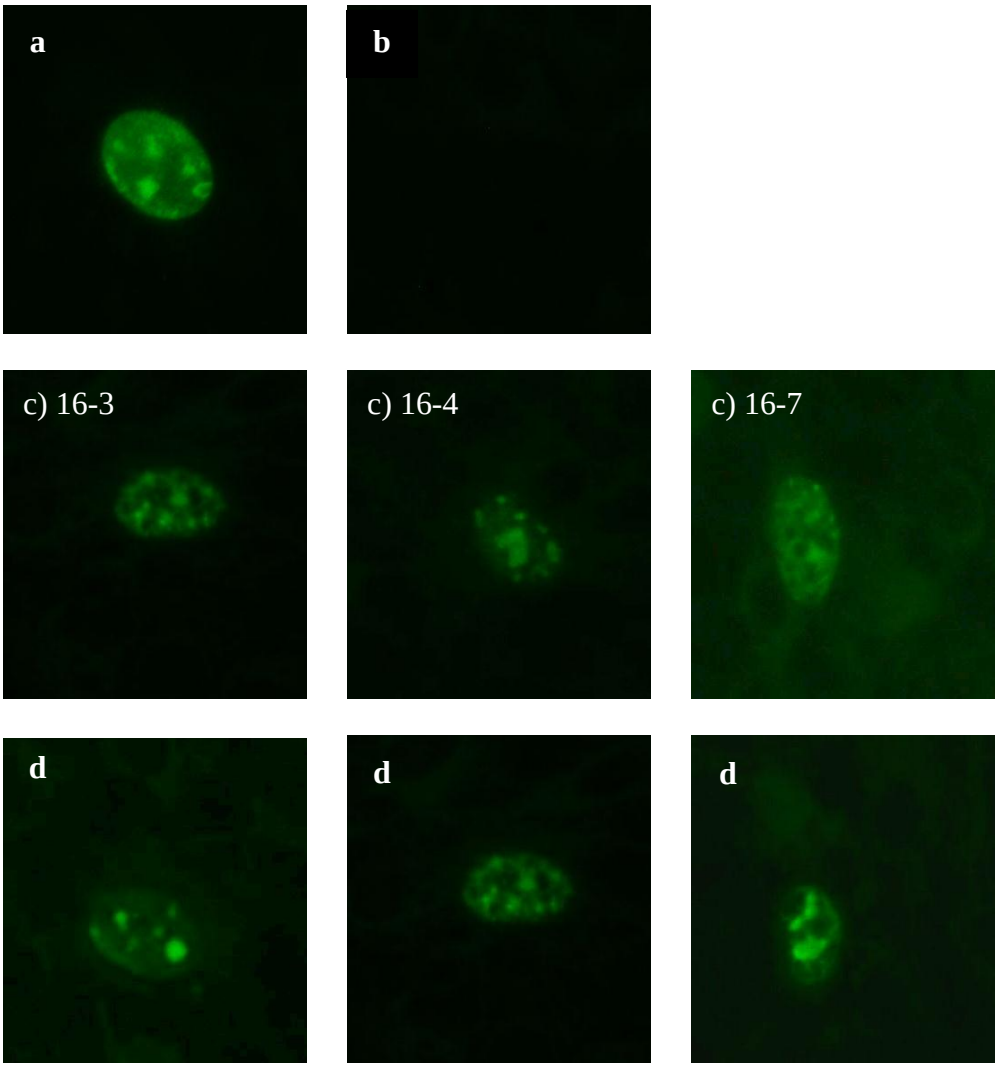
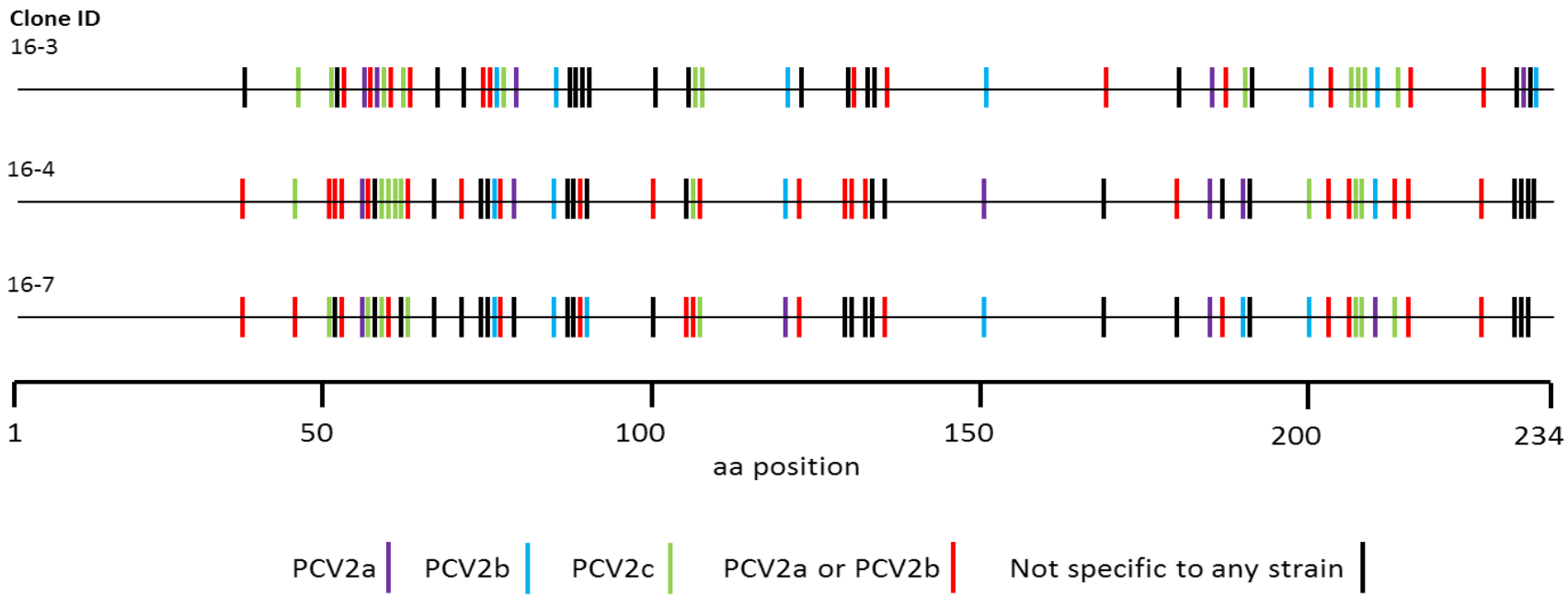


Figure 2.5: Capsid genetic makeup of viable infectious individual PCV2-S viruses recovered from PK-15 cells. (a) Diagram of positions of variation; (b) putative amino acid sequence in position of variation of the three PCV2-S viruses.

a



b

aa position	39	47	52	53	54	57	58	59	60	61	63	64	68	72	75	76	77	78	80	86	88	89	90	91	101	106	107	108	121	123	130	131	133
16-3	R	A	T	F	G	V	K	G	S	Q	S	T	T	M	K	L	N	D	V	S	H	S	S	L	V	C	A	C	S	V	V	T	A
16-4	R	T	S	L	G	V	N	K	S	T	G	P	N	L	T	F	N	D	F	S	H	S	S	V	I	W	P	R	T	V	F	P	T
16-7	K	A	S	L	G	V	K	A	S	T	S	T	S	L	N	I	N	Q	V	S	N	H	P	L	I	C	A	R	S	I	I	T	T

134	136	151	169	180	185	187	190	191	200	203	206	207	208	210	213	215	226	231	232	233	234	235
S	Q	P	C	R	M	I	S	K	H	E	I	N	A	E	I	V	L	I	Y	L	K	STOP
N	L	T	W	K	M	L	A	A	I	E	I	N	A	D	V	V	L	I	N	R	STOP	
D	L	T	S	K	M	L	T	E	I	E	T	N	A	E	V	V	L	I	K	L	L	STOP

PCV2a | PCV2a or PCV2b | PCV2b | PCV2c | Not specific to any subtype

Figure 2.6: Chimeric PCV2-S-HA diagram and IFA results. (a) Diagram of HA tag insertion location. IFA pictures of PCV2-S-HA library using FITC-labeled anti-HA monoclonal antibody: (b) Nuclear staining of PK-15 cells transfected with PCV2-S-HA DNA library; (c) Nuclear staining of PK-15 cells infected with the PCV2-S-HA library. All pictures at 200x magnification.

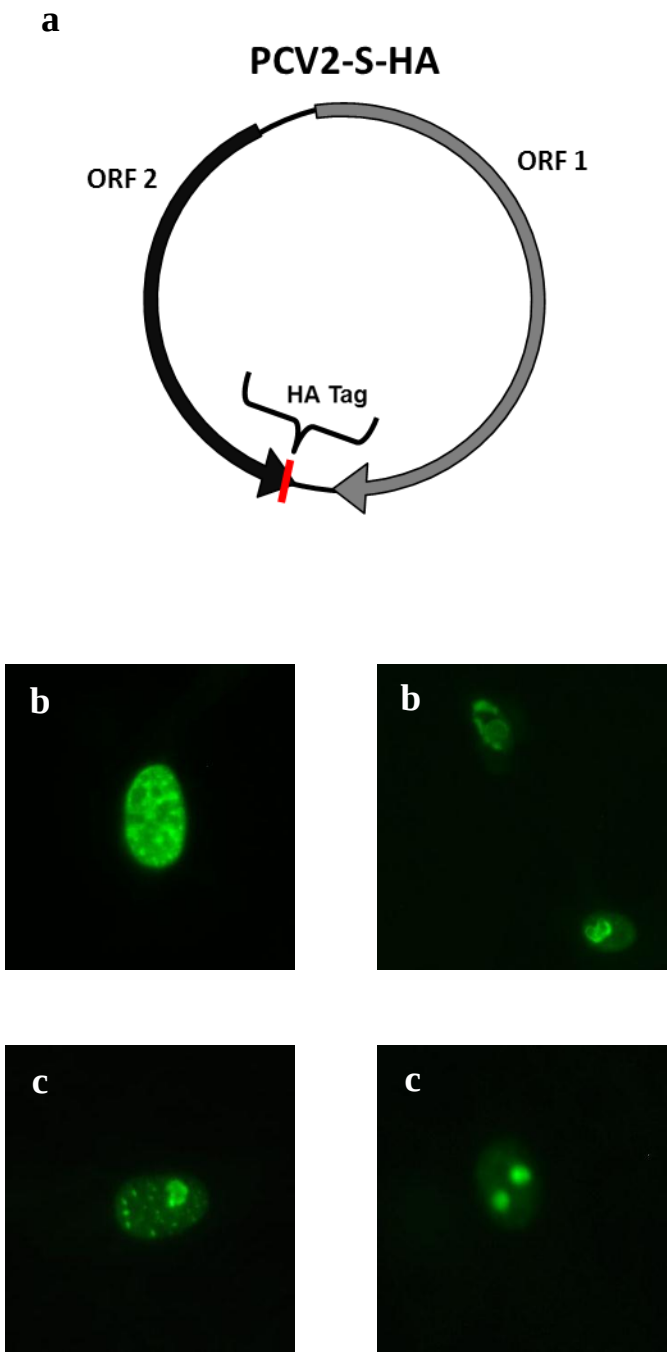


Table 2.1: Oligonucleotides used to generate shuffled PCV2 capsid gene sequences

Oligo Name	Nucleotide Sequence (5'-3')
Forward*	
fA†	ATGACGTATCCAAGGAGGCGTTACCGCAGAAGAAGACACCGCCCCCGCAG
fB	TCCTCCGCCGCCGCCCCTGGCTCGTCCACCCCCGCCACCGCTACCGTTGGARAAGGAAAAATG
fC1	CCGCWCCHTCGKATATACTDTCARGAVGWCCACAGTCAVAMCGCCCTCCTGG
fC2	CCGCWCCHTCGKATATACTDTCAATAVGWCCACAGTCAVAMCGCCCTCCTGG
fC3	CCGCWCCHTCGKATATACTDTCARGGSTWCCACAGTCAVAMCGCCCTCCTGG
fC4	CCGCWCCHTCGKATATACTDTCAATGSTWCCACAGTCAVAMCGCCCTCCTGG
fC5	CCGCWCCHTCGKATATACTDTCARGAVGWCCCAGGTCAVAMCGCCCTCCTGG
fC6	CCGCWCCHTCGKATATACTDTCAATAVGWCCCAGGTCAVAMCGCCCTCCTGG
fC7	CCGCWCCHTCGKATATACTDTCARGGSTWCCCAGGTCAVAMCGCCCTCCTGG
fC8	CCGCWCCHTCGKATATACTDTCAATGSTWCCCAGGTCAVAMCGCCCTCCTGG
fC9	CCGCWCCHTCGKATATACTDTCARGAVGWCCACAGTCDGTMCGCCCTCCTGG
fC10	CCGCWCCHTCGKATATACTDTCAATAVGWCCACAGTCDGTMCGCCCTCCTGG
fC11	CCGCWCCHTCGKATATACTDTCARGGSTWCCACAGTCDGTMCGCCCTCCTGG
fC12	CCGCWCCHTCGKATATACTDTCAATGSTWCCACAGTCDGTMCGCCCTCCTGG
fC13	CCGCWCCHTCGKATATACTDTCARGAVGWCCCAGGTCDGTMCGCCCTCCTGG
fC14	CCGCWCCHTCGKATATACTDTCAATAVGWCCCAGGTCDGTMCGCCCTCCTGG
fC15	CCGCWCCHTCGKATATACTDTCARGGSTWCCCAGGTCDGTMCGCCCTCCTGG
fC16	CCGCWCCHTCGKATATACTDTCAATGSTWCCCAGGTCDGTMCGCCCTCCTGG
fD1	TTCCCCCGGGAGGGGGGWCCAACAAWAKCHCTVTACCCTTTGAATACTACAGAATAAGAAAG
fD2	TTCCCCCGGGAGGGGGGWCCAACCMCAKCHCTVTACCCTTTGAATACTACAGAATAAGAAAG
fD3	TTCCCCCGGGAGGGGGGWCCAACAAWCDTHCTVTACCCTTTGAATACTACAGAATAAGAAAG
fD4	TTCCCCCGGGAGGGGGGWCCAACCMCCDTHCTVTACCCTTTGAATACTACAGAATAAGAAAG
fE	CCYGCTCCCCCATCACCCAGGGTGATAGGGGAGTGGGCTCCASTGCTRTTATTCTAGATGATAACT
fF	GCCCWAACCTATGACCCATATGTAAACTACTCCTCCCGCCATACAATCMCCCAACCCTTCTCCTACCACT
fG1	CCCAAACCTGTTCTTGACTSGACCATTGATTACTTCCAACCAAATAACAAAARAAATCAGCTTTGG
fG2	CCCAAACCTGTTCTTGACBGCACCATTGATTACTTCCAACCAAATAACAAAARAAATCAGCTTTGG
fH1	CCACGTAGGCCTCGGCCATGCGTTCSAAAACAGTAHAWACGMCCAGGASTACAATRTCCGTRTAACCATGTATGTACA
fH2	CCACGTAGGCCTCGGCCATGCGTTCSAAAACAGTAHAWACGMCCAGGCCTACAATRTCCGTRTAACCATGTATGTACA
fH3	CCACGTAGGCCTCGGCAYTGCGTTCSAAAACAGTAHAWACGMCCAGGASTACAATRTCCGTRTAACCATGTATGTACA
fH4	CCACGTAGGCCTCGGCAYTGCGTTCSAAAACAGTAHAWACGMCCAGGCCTACAATRTCCGTRTAACCATGTATGTACA
fJ1	AATYTTAAAGACCCCCCABTTAAWCNCHAATAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
fJ2	AATYTTAAAGACCCCCCAAKTAAWCNCHAATAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA

Table 2.1 continued: Oligonucleotides used to generate shuffled PCV2 capsid gene sequences

Oligo Name	Nucleotide Sequence (5'-3')
fJ3	AATYTTAAAGACCCCCCABTTYATCNCHAATAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
fJ4	AATYTTAAAGACCCCCCAAKTYATCNCHAATAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
fJ5	AATYTTAAAGACCCCCCABTTAAWCNCTTGTAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
fJ6	AATYTTAAAGACCCCCCAAKTAAWCNCTTGTAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
fJ7	AATYTTAAAGACCCCCCABTTYATCNCTTGTAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
fJ8	AATYTTAAAGACCCCCCAAKTYATCNCTTGTAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
TLNE1	AATYTTAAAGACCCCCCABTTACGCTGAATGAGTAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
TLNE2	AATYTTAAAGACCCCCCAAKTACGCTGAATGAGTAAATGAATAATAAAAACCATTACGAAGTGATAAAAAAGACTCA
QMKN1	AATYTTAAAGACCCCCCABTTAAWCNCCAAATGAAGAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
QMKN2	AATYTTAAAGACCCCCCAAKTAAWCNCCAAATGAAGAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
QMKN3	AATYTTAAAGACCCCCCABTTYATCNCCAAATGAAGAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
QMKN4	AATYTTAAAGACCCCCCAAKTYATCNCCAAATGAAGAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
LMNN1	AATYTTAAAGACCCCCCABTTAAWCNCCTAATGAATAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
LMNN2	AATYTTAAAGACCCCCCAAKTAAWCNCCTAATGAATAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
LMNN3	AATYTTAAAGACCCCCCABTTYATCNCCTAATGAATAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
LMNN4	AATYTTAAAGACCCCCCAAKTYATCNCCTAATGAATAATAAAAACCATTACTAAGTGATAAAAAAGACTCA
End of fJ	GTGATAAAAAAGACTCAGTA
fJ end HA‡	TACCCATACGATGTTCCAGATTACGCTTAAATGAATAATAAAAACCATTA
fJ1 - HA	AATYTTAAAGACCCCCCABTTAAWCNCHAATACCCATACGATGTTCCAGATTACGCT
fJ2 - HA	AATYTTAAAGACCCCCCAAKTAAWCNCHAATACCCATACGATGTTCCAGATTACGCT
fJ3 - HA	AATYTTAAAGACCCCCCABTTYATCNCHAATACCCATACGATGTTCCAGATTACGCT
fJ4 - HA	AATYTTAAAGACCCCCCAAKTYATCNCHAATACCCATACGATGTTCCAGATTACGCT
fJ5 - HA	AATYTTAAAGACCCCCCABTTAAWCNCTTGTACCCATACGATGTTCCAGATTACGCT
fJ6 - HA	AATYTTAAAGACCCCCCAAKTAAWCNCTTGTACCCATACGATGTTCCAGATTACGCT
fJ7 - HA	AATYTTAAAGACCCCCCABTTYATCNCTTGTACCCATACGATGTTCCAGATTACGCT
fJ8 - HA	AATYTTAAAGACCCCCCAAKTYATCNCTTGTACCCATACGATGTTCCAGATTACGCT
TLNE1 - HA	AATYTTAAAGACCCCCCABTTACGCTGAATGAGTACCCATACGATGTTCCAGATTACGCT
TLNE2 - HA	AATYTTAAAGACCCCCCAAKTACGCTGAATGAGTACCCATACGATGTTCCAGATTACGCT
Reverse*	
rA	CCCCATGCCCTGAATTTCCATATGAAATAAATTACTGAGTCTTTTTTATCACTT
rB	TGGGGGGTCTTTAARATTAAATTCTCTGAATTGTACATACATGGTTA
rC1	GCCGAGGCCTACGTGGTCCACATTTYTAGHGGTTTGTABCCTCAWCCAAAGCTGATTTYTTTTGTTATT

Table 2.1 continued: Oligonucleotides used to generate shuffled PCV2 capsid gene sequences

Oligo Name	Nucleotide Sequence (5'-3')
rC2	GCCGAGGCCTACGTGGTCCACATTCBCAGHGGTTTGTABCCTCAWCCAAAGCTGATTTYTTTTGTTATT
rD	GTCAAGAACAGGTTTGGGTGTGAAGTAACGGGAGTGGTAGGAGAAGGGTTGGG
rE1	ACATATGGGTCATAGGTTWGGGCTGNGRCCTTTGKAAHAAAGTTATCATCTAGAATAA
rE2	ACATATGGGTCATAGGTTWGGGCTGNGRCCTTYATAAHAAAGTTATCATCTAGAATAA
rE3	ACATATGGGTCATAGGTTWGGGCTGNGSTCTTTGKAAHAAAGTTATCATCTAGAATAA
rE4	ACATATGGGTCATAGGTTWGGGCTGNGSTCTTYATAAHAAAGTTATCATCTAGAATAA
rE5	ACATATGGGTCATAGGTTWGGGCGTYGRCCTTTGKAAHAAAGTTATCATCTAGAATAA
rE6	ACATATGGGTCATAGGTTWGGGCGTYGRCCTTYATAAHAAAGTTATCATCTAGAATAA
rE7	ACATATGGGTCATAGGTTWGGGCGTYGSTCTTTGKAAHAAAGTTATCATCTAGAATAA
rE8	ACATATGGGTCATAGGTTWGGGCGTYGSTCTTYATAAHAAAGTTATCATCTAGAATAA
rF1	GTGATGGGGGAGCRGGSCCRGAATTCAACCTTAAYCTTTCTTATTCTGTAGTATTC
rF2	GTGATGGGGGAGCRGGSAMAGAATTCAACCTTAAYCTTTCTTATTCTGTAGTATTC
rG1	CCCCCCTCCCGGGGGAAVAAAGTCGTYGANMTTAAATCTCAWCATGTCCACCGMCCAGGAGGGCG
rG2	CCCCCCTCCCGGGGGAAVAAAGTCGTYGANMTTAAATCTCAWCATGTCCACAKTCCAGGAGGGCG
rG3	CCCCCCTCCCGGGGGAAVAAAGTCGTYGANAVTAAATCTCAWCATGTCCACCGMCCAGGAGGGCG
rG4	CCCCCCTCCCGGGGGAAVAAAGTCGTYGANAVTAAATCTCAWCATGTCCACAKTCCAGGAGGGCG
rG5	CCCCCCTCCCGGGGGAAVAAACTGGTYGANMTTAAATCTCAWCATGTCCACCGMCCAGGAGGGCG
rG6	CCCCCCTCCCGGGGGAAVAAACTGGTYGANMTTAAATCTCAWCATGTCCACAKTCCAGGAGGGCG
rG7	CCCCCCTCCCGGGGGAAVAAACTGGTYGANAVTAAATCTCAWCATGTCCACCGMCCAGGAGGGCG
rG8	CCCCCCTCCCGGGGGAAVAAACTGGTYGANAVTAAATCTCAWCATGTCCACAKTCCAGGAGGGCG
rH	AHAGTATATMCGADGGWGCGGGAGAGGCGGGHGTTGAAGATGCCATTTTTCCTTYTCCAACGGTAG
rJ	GCCAGGGGCGGCGGCGGAGGATCTGGCCAAGATGGCTGCGGGGGCGGTGTCTTCTT
BsmI site†	TTGGAAGAATGCTACAGAAC

* Direction relative to PCV2 capsid

†Primers used to amplify capsid following DNA extraction from PK-15 cells

‡Primers with '-HA' used to add HA tag to C' end of shuffled capsid

Table 2.2: Positions of variation and corresponding amino acids represented within the library of shuffled PCV2 capsid sequences. Numbers in parentheses indicate the percentage of isolates published on GenBank containing that specific amino acid.

Position*	amino acids (percentage)						Position	amino acids					
39	$R_{(98.7)}$	$K_{(1.3)}$					**121**	$S_{(80.3)}$	$T_{(19.7)}$				
47	$T_{(94.7)}$	$A_{(5.2)}$	$S_{(0.1)}$				**123**	$V_{(94.6)}$	$I_{(5.4)}$				
52	$T_{(99.4)}$	$S_{(0.6)}$					**130**	$V_{(95.9)}$	$F_{(3.2)}$	$I_{(0.6)}$			
53	$F_{(94.7)}$	$I_{(5.0)}$	$L_{(0.3)}$				**131**	$T_{(84.9)}$	$P_{(10.9)}$	$I_{(3.8)}$	$M_{(0.3)}$		
54	$V_{(99.5)}$	$G_{(0.5)}$					**133**	$A_{(95.0)}$	$S_{(3.1)}$	$V_{(1.5)}$	$T_{(0.4)}$		
57	$I_{(63.5)}$	$V_{(36.4)}$	$F_{(0.1)}$				**134**	$T_{(89.4)}$	$N_{(8.6)}$	$P_{(1.2)}$	$A_{(0.5)}$	$S_{(0.2)}$	$D_{(0.1)}$
58	$K_{(98.6)}$	$N_{(0.5)}$	$R_{(0.3)}$				**136**	$L_{(93.5)}$	$Q_{(6.5)}$				
59	$R_{(78.9)}$	$A_{(17.7)}$	$K_{(2.6)}$	$G_{(0.7)}$	$T_{(0.1)}$		**151**	$T_{(76.5)}$	$P_{(23.5)}$				
60	$T_{(98.5)}$	$S_{(1.5)}$					**169**	$S_{(90.9)}$	$R_{(6.9)}$	$G_{(2.0)}$	$W_{(0.1)}$	$C_{(0.1)}$	
61	$T_{(99.4)}$	$Q_{(0.5)}$					**180**	$R_{(98.3)}$	$K_{(1.5)}$	$I_{(0.1)}$			
63	$K_{(47.5)}$	$R_{(36.2)}$	$T_{(10.7)}$	$S_{(5.3)}$	$G_{(0.2)}$	$C_{(0.1)}$	**185**	$L_{(92.9)}$	$M_{(7.1)}$				
64	$T_{(99.3)}$	$P_{(0.7)}$					**187**	$L_{(97.2)}$	$I_{(2.7)}$	$V_{(0.1)}$			
68	$A_{(95.4)}$	$N_{(3.7)}$	$S_{(0.8)}$	$T_{(0.1)}$			**190**	$A_{(63.9)}$	$S_{(19.6)}$	$T_{(16.5)}$			
72	$M_{(92.5)}$	$L_{(7.5)}$					**191**	$G_{(80.5)}$	$R_{(13.3)}$	$A_{(4.7)}$	$K_{(1.3)}$	$E_{(0.2)}$	
75	$N_{(90.9)}$	$K_{(8.5)}$	$T_{(0.4)}$	$S_{(0.1)}$	$I_{(0.1)}$		**200**	$T_{(98.6)}$	$I_{(0.5)}$	$H_{(0.5)}$			
76	$I_{(89.0)}$	$L_{(10.7)}$	$F_{(0.2)}$	$V_{(0.1)}$			**203**	$E_{(99.3)}$	$Q_{(0.5)}$				
77	$N_{(81.7)}$	$D_{(18.3)}$					**206**	$I_{(80.0)}$	$K_{(19.1)}$	$T_{(0.6)}$			
78	$D_{(99.4)}$	$Q_{(0.5)}$					**207**	$Y_{(99.5)}$	$N_{(0.5)}$				
80	$L_{(79.5)}$	$V_{(19.8)}$	$F_{(0.1)}$				**208**	$D_{(99.3)}$	$A_{(0.7)}$				
86	$S_{(79.5)}$	$T_{(20.5)}$					**210**	$E_{(72.1)}$	$D_{(27.3)}$	$A_{(0.6)}$			
88	$P_{(79.1)}$	$K_{(20.7)}$	$N_{(0.1)}$	$H_{(0.1)}$			**213**	$I_{(99.4)}$	$V_{(0.6)}$				
89	$R_{(72.2)}$	$I_{(20.7)}$	$L_{(6.9)}$	$H_{(0.1)}$	$S_{(0.1)}$		**215**	$V_{(94.5)}$	$I_{(5.5)}$				
90	$S_{(92.9)}$	$T_{(6.9)}$	$P_{(0.2)}$				**226**	$L_{(99.0)}$	$F_{(0.8)}$				
91	$V_{(79.3)}$	$I_{(20.5)}$	$L_{(0.1)}$				**231**	$L_{(98.9)}$	$S_{(0.5)}$	$F_{(0.2)}$	$V_{(0.2)}$	$I_{(0.1)}$	
101	$V_{(98.5)}$	$I_{(1.5)}$					**232**	$N_{(77.2)}$	$K_{(20.5)}$	$H_{(0.9)}$	$Y_{(0.4)}$		
106	$W_{(98.9)}$	$C_{(0.4)}$	$F_{(0.4)}$	$R_{(0.2)}$			**233**	$P_{(98.6)}$	$L_{(0.5)}$	$R_{(0.1)}$	$H_{(0.1)}$		
107	$P_{(99.4)}$	$A_{(0.6)}$					**234**	$K_{(98.1)}$	$Q_{(0.8)}$	$L_{(0.5)}$	$R_{(0.3)}$	$N_{(0.3)}$	
108	$C_{(99.3)}$	$R_{(0.5)}$											

*Position relative to PCV2 capsid, position 1 corresponds to start codon

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