Genetic Stability of a Genetically-Engineered Chimeric Porcine Circovirus (PCV) Vaccine, PCV1-2

By

Jennifer Ann Gillespie

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Xiang-Jin Meng, Chair
F. William Pierson
Stephen M. Boyle

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Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of porcine circovirus associated disease (PCVAD), an economically important swine disease that causes wasting in pigs 5-18 weeks of age. There exist two different types of porcine circoviruses: porcine circovirus type 1 (PCV1) was discovered as a contaminant of porcine kidney (PK-15) cells and was determined to be nonpathogenic in swine; whereas porcine circovirus type 2 (PCV2) is pathogenic. A recently released vaccine for PCVAD was generated by inserting the gene encoding the immunogenic capsid protein of PCV2 into the genetic backbone of the non-pathogenic PCV1. This chimeric PCV vaccine, called PCV1-2, was shown to induce protective immunity against PCV2 infection in pigs. The vaccine is currently on the market in a killed form. In order to develop a live version of the vaccine, the genetic stability of the chimeric PCV1-2 vaccine virus was investigated by *in vitro* and *in vivo* passaging of the vaccine virus. *In vitro* passaging of the PCV1-2 vaccine virus was done in a porcine kidney PK-15 cell line. Cells were infected with the PCV1-2 vaccine virus and then serially passaged 11 times. The passaged vaccine viruses recovered from passages 5 and 11 were sequenced, and the sequences were compared to that of the original PCV1-2 vaccine virus. The *in vitro* serial passage result showed that no mutation occurred during the 11 *in vitro* passages. The *in vivo* passaging was done using specific-pathogen-free (SPF) pigs. In *in vivo*

"passage 1", nine piglets were divided into 3 groups of 3 each: group 1 each inoculated with 200ug of PCV1-2 plasmid, group 2 each with 1×10^3 TCID$_{50}$ live PCV1-2 vaccine virus, and group 3 each with 3ml phosphate buffered saline (PBS) buffer as a control. One pig from each group was necropsied at 14, 21, and 28 days post-inoculation (DPI), respectively. A panel of tissue samples including lymph nodes and thymus were collected from each pig. Tissue homogenates from DPI 28 that were positive by PCR for PCV1-2 DNA were used to inoculate new piglets in the *in vivo* passage 2 experiment. Viruses recovered from passage 2 pigs were subsequently used for inoculation in the *in vivo* passage 3 experiment. The PCV1-2 vaccine virus DNA from pigs in each passage was amplified and sequenced. The results of the *in vivo* serial passage experiment showed that, after 3 passages of the PCV1-2 vaccine virus in pigs, there were no new mutations in the viruses recovered from pigs. The PCV1-2 vaccine contained an introduced marker mutation at amino acid position number 79, which is in the capsid region. During the *in vivo* passaging of the vaccine virus in pigs, this marker mutation quickly reverted back to its original nucleotide. This marker back mutation occurred between DPI 21 and DPI 28 of passage 1 in the PCV1-2 live vaccine virus group, and between DPI 28 of passage 1 and DPI 14 of passage 2 in the PCV1-2 vaccine plasmid group, and remained stable throughout the reminder of the *in vivo* study. Based upon the results from this study, we conclude that the PCV1-2 chimeric vaccine virus is genetically stable *in vitro* and in pigs, and thus should serve as a good candidate for a live vaccine against PCV2.

This thesis is dedicated to my family and friends. For the love and support from my parents, Mary and Charles Gillespie, my brothers Mike, Andrew and John, my caring and patient fiancé Jeff, and my veterinary mentors Dr. and Mrs. Anna Bruce

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Abbreviations

AASV	American Association of Swine Veterinarians
Bp	Base pairs
DNA	Deoxyribonucleic Acid
DPI	Days post infection
ELISA	Enzyme Linked Immunosorbant Assay
GAG	Glucose Aminoglycans
ICH	Immunohistochemistry
IL	Interleukin
IM	Intramuscular
INF_a	Interferon Alpha
IPMA	IgM immunoperoxidase monolayer assay
ISH	*in situ* hybridization
IV	Intravenous
Kb	Kilobases
mL	Milliliter
Nt	Nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PCV	Porcine Circovirus
PCV1	Porcine Circovirus Type 1
PCV2	Porcine Circovirus Type 2
PCVAD	Porcine Circovirus Associated Disease
PDND	Porcine Dermatitis and Nephropathy Syndrome
PMWS	Post-Weaning Multisystemic Wasting Disease
PPV	Porcine Parvovirus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RNA	Ribonucleic Acid
SPF	Specific Pathogen Free
ssDNA	Single Stranded DNA
$TCID_{50}$	50% Tissue culture infectious dose
UK	United Kingdom
uL	Microliter
USDA	United States Department of Agriculture

General Introduction

Porcine circovirus type 1 (PCV1) was originally discovered as a persistent contaminant of the porcine kidney PK-15 cell line (ATCC CCL-33). This virus does not cause clinical disease and was determined to be nonpathogenic in swine. Porcine circovirus type 2 (PCV2) was discovered in association with a disease known as porcine circovirus associated disease (PCVAD), formally known as postweaning multisystemic wasting syndrome (PMWS), an economically very important disease that causes wasting in weaning piglets. It was determined that this virus is the primary causative agent of PCVAD.

PCV1 and PCV2 both belong to the family *Circoviridae*. Also in this group are chicken anemia virus and psittacine beak and feather disease viruses. They have a small, non-enveloped icosahedral virion with a single stranded, circular DNA genome about 1.76 kb in size which contains 2 major open reading frames (ORFs). ORF1 encodes for viral replication proteins, ORF2 encodes for the immunogenic capsid protein. PCV1 and PCV2 share approximately 76% nucleotide sequence homology and are similarly organized. PCV2 is a ubiquitous swine pathogen and has been found in almost all of the major swine producing countries.

PCVAD is a devastating swine disease which was first discovered in weaning piglets in Canada in 1991 and now affects all major swine producing countries. This disease first affects pigs between 5 and 18 weeks old. Pigs contract the disease through nasal and fecal shedding and mortality can reach up to 50%. Clinical signs of PCVAD are wasting with progressive weight loss, lethargy, diarrhea, lymphadenopathy, and

jaundice. The major histopathologic lesions are lymphoid depletion with histiocytic replacement. Co-infection with other pathogens such as porcine parvovirus (PPV) and porcine reproductive and respiratory syndrome virus (PRRSV) has been shown to cause a more severe disease course.

A recently available vaccine against PCV2 and PCVAD was generated by inserting the gene encoding the immunogenic capsid protein of PCV2 into the genetic backbone of nonpathogenic PCV1. This vaccine, called PCV1-2, is attenuated in pigs and induces protective immunity against PCV2 infection in pigs. It is currently available in a killed form, and to produce a live version of this vaccine, testing must be done to determine the genetic stability of the PCV2 genome in animals. Live vaccines are preferable since they generally induce both humoral and cell-mediated immune responses. However, they have the potential to mutate or revert to a pathogenic state. The objective of my thesis research is to determine the *in vitro* and *in vivo* genetic stability of the chimeric PCV1-2 vaccine.

Chapter 1

Literature Review

1.1 Introduction

Porcine circovirus type 2 (PCV2) is the primary causative agent of several syndromes collectively known as porcine circovirus associated disease (PCVAD). PCVAD is a globally emerging disease that is having a huge impact on swine producing countries and is arguably the most economically important disease affecting the global swine industry today. The British Pig Executive national herd mortality data for finisher pigs in 2006 found that mortality increased from 3.3% to 6.5% when PCVAD began affecting herds. Fifty percent of affected farms had mortality rates over 9.7%. (British Pig Executive (BPEX). 2006 *Pig Yearbook 2006*;47-50).

PCV2 infection is present in every major swine producing country in the world and the number of cases of PCVAD is rapidly increasing. A report from the Ontario Animal Health Surveillance Network, published in July 2005, indicated that in 1998, less than 20 cases were reported. In contrast, 150 cases were reported during the first half of 2005 and these numbers continue to increase.

In the United States, the disease has cost producers an average of 3-4 dollars per pig with peak losses ranging up to 20 dollars per pig. The importance of this disease has stimulated investigations aimed at identifying risk factors associated with infection and minimizing these risks through modified management practices and development of

vaccination strategies. This paper provides an overview of current knowledge relating to PCV2 and PCVAD with an emphasis on information relevant to the swine veterinarian.

1.2 History of the Porcine Circovirus (PCV)

There exist two phenotypically different but genetically related strains of porcine circoviruses [1], [2]. Porcine circovirus type 1 (PCV1) was originally discovered in 1974 as a persistent contaminant of the porcine kidney PK-15 cell line ATCC CCL-33 [3]. This virus is widespread in the swine population but does not cause clinical disease and is nonpathogenic in swine [4], [5], [6]. Porcine circovirus type 2 (PCV2) was discovered in association with postweaning multisystemic wasting syndrome (PMWS) in Canadian weaning piglets in 1991 [5], [7] , [8], [9]. It is the smallest known freely replicating virus in vertebrates [10]. PCV2 has been recognized as the primary causative agent of PMWS [11], now known as porcine circovirus associated disease (PCVAD) since the name was modified in March 2006 by the American Association of Swine Veterinarians.

After Harding and Clark defined the syndrome in 1997 [9], it became clear that PCVAD was the cause of many losses in pig herds in all the major swine producing countries [1], [5], [8], [12], [11], [13]. Retrospective studies of pig serum samples found PCV2-specific antibodies as early as 1969 in Belgium [14], 1970 in the United Kingdom [13], 1973 in Ireland [15], and 1985 in Canada and Spain [16], [17]. PCV2 antibodies were also identified in 13.6% of tissues collected from Canadian pigs in 1985. Virus positive samples increased to 72.4% in 1989, and then leveled off at 66.7% in 1997 [16]. In Northern Ireland, 69.1% of serum samples were antibody positive in 1973, 55% in 1984, 100% in 1988, and 92.1% in 1999 [15].

Retrospective studies on archived pig tissues in the United Kingdom diagnosed PCVAD in 68 cases between 1970 and 1997 [13]. Sequence analysis of the virus from those tissues showed a high sequence identity to isolates from a pig diagnosed in the year 2000 with another disease associated with PCV2, porcine dermatitis and nephropathy syndrome (PDNS). This indicated that the virus changed very little during the 30 year period [13]. Serology has demonstrated that PCV2 antibodies are present globally in most swine herds and up to 100% of individual pigs within those herds [15], [16], [18]; this includes herds in the United States [18].

1.3 Current State of PCVAD

PCVAD is considered an emerging disease and the incidence of this disease has increased dramatically over the past years. In Part II of the Reference of Swine Health and Health Management in the United States published in the year 2000 by the USDA [19], PCVAD prevalence was determined by USDA veterinarians who collected data from commercial herds of 100 or more pigs accounting for nearly 94% of the United States pig inventory (Table 1).

Of nursery age pigs, the percentage of sites where PCVAD was known or suspected to have caused sickness or mortality in one or more pigs during the previous 12 months in farms with <2000 animals was 4.4%, farms with 2000-10,000 animals had 10.4%, and >10,000 animals reported 20.9% for an overall of 5.7%. Approximately 30% of animals were diagnosed by a veterinarian or diagnostic laboratory. In grower and finisher pigs, small farms had 2.3%, medium farms had 8.8% and large farms had 12.4%

affected pigs for an overall prevalence of 3.6%. Approximately 54% of the pigs reported to have PCVAD were diagnosed by a veterinarian or diagnostic laboratory.

When the same report was published in the year 2006, which again accounted for 94% of the United States pig population [20], prevalence in nursery pigs on small farms (<2000 animals) was 21.5%, medium farms (2000-5000 animals) was 12.5%, and large farms (>5000 animals) was 39.6% for an overall of 22.3%. Approximately 60% were diagnosed by a veterinarian. In growers and finishers, small farms reported 25.0%, medium farms 35.4%, and large farms 59.9% for an overall of 31.3%. Approximately 70% were diagnosed by a veterinarian or diagnostic laboratory.

The prevalence of porcine dermatologic and nephropathy syndrome was also described for the first time in the 2006 report, and in nursery pigs of small farms the prevalence was 3.3%, medium farms 0.0%, and large farms 3.4% for an overall of 2.9%. In growers and finishers the numbers were 1.6% for small, 10.4% for medium and 23.9% for large farms, overall prevalence was 6.0%. The age of onset for PCVAD affected pigs ranged from 8.9-16.3 weeks (Table 1).

1.4 Taxonomy

PCV1 and PCV2 belong to the family *Circoviridae* [5], [8], [11], [21] and to the genus *Circovirus* [22]. Other known viruses in this genus are canary circovirus, goose circovirus, pigeon circovirus and psittacine beak and feather disease virus. Another genus in the circoviridae family, *Gyrovirus*, includes chicken anemia virus [22]. The *Gyrovirus* genus is distinct in that it has a negative sense genome and larger virions than is typical for circovirus [23]. Circoviruses are host specific, most of which are avian, or have a

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relatively narrow host range. Several species produce lymphoid depletion in infected hosts while others cause subclinical infection [22]. PCV1 is most closely related to the beak and feather disease virus [24]. Several human circoviruses also exist and include the torque teno virus (TTV) which is related to the swine TTV, TTV-like mini virus, and the SEN virus. The human circoviruses have not been definitively linked to any disease in humans [25], [26], [27], [28].

Circoviridae is most closely related to the family Nanoviridae which includes plant viruses. These viruses share a step loop structure at the origin of replication and show similarities in the replication proteins [29]. These similarities are also shared by the plant Geminiviruses, and it is speculated that circovirus may be the genetic link between the two plant virus families [24]. It has been proposed that an ancestor of PCV1 may have been a plant nanovirus that infected a vertebrate host and recombined with a vertebrate-infecting RNA virus which was most likely a calicivirus [30].

PCV2 has been divided into two distinct genotypes. They have been named PCV2-group 1 and PCV2-group 2 [2]. There is no difference in pathogenesis between the two genotypes, but the viruses differ in size with PCV2-group 1 being 1767 nucleotides (nt) and PCV2-group 2 being 1768 nt [2]. PCV2-group 1 is further divided into 3 clades and PCV2-group 2 divided into 5 clades [2]. Historically, only PCV2-group 2 isolates were found in the United States; but in late 2005 several outbreaks of higher than normal mortality (5-50%) were reported in Kansas, North Carolina and Iowa. These outbreaks were found to be associated with PCV2-group 1 isolates [31].

About the same time as PCV2 was being grouped in to group 1 and group 2 isolates, North American laboratories proposed grouping PCV2 into North American

isolates, or PCV2a, and European like isolates, or PCV2b 32. PCV2b falls into PCV2-group 1 and PCV2a falls into PCV2-group 2 [32].

1.5 Genomic organization

PCV 1 and PCV 2 have a small, non-enveloped icosahedral virion 3 with a single stranded, circular DNA genome of 1759 nt and 1767-1768 nt in size, respectively [1], [29]. They contain 2 major open reading frames (ORFs) encoded in an antisense direction [22] (Figure 1). ORF1 encodes for viral replication proteins (Rep), and ORF2 encodes for the capsid protein (Cap), which contains the immunodominant antigenic epitopes [29], [33], [34], [35], [36]. ORF 1 is very similar between PCV1 and PCV2 with 83% nucleotide and 86% amino acid identity between them [37]. ORF 1 is alternatively spliced into 8 RNA strands in PCV1 and 5 RNA strands in PCV2. Only two RNA strands, Rep and Rep' are essential for virus replication [34], [38], [39]. PCV1 and PCV2 share 67% nucleotide and 65% amino acid sequence identity in ORF 2 [37]. A third ORF has been described in PCV2 and it has been suggested that ORF 3 is involved with apoptosis [40], but this report has not been able to be verified by independent laboratories. Overall, PCV1 and PCV2 share 76% nucleotide sequence homology and are similarly organized [1], [41].

1.6 Virus Life Cycle

The mechanisms of PCV2 cell recognition, attachment and entry are currently being researched and not well understood. It is believed that PCV2 uses a relatively common cell receptor, since viral replication and PCV2 antigen has been found in many

different cell types [42]. PCV2 binds to heparin sulfate and chondroitin sulfate, which are glycosaminoglycans (GAGs), as a first step of attachment [43]. However, as PCV2 is found in cells that lack GAGs, it is thought that another co-receptor is also used for viral entry [43].

The hallmark lesion of PCV2 infection is lymphoid depletion with histiocytic replacement. In affected lymph organs, dendritic cells and macrophages that replace the lymphocytes contain large amounts of PCV2 virus [5], [44], [45]. There is no viral degradation in these cells, and since dendritic cells are highly mobile, it is thought that dendritic mobility may be a method of viral dissemination in tissues [46]. It is still unknown how PCV2 causes a reduction in lymphocytes. Hypotheses include induced apoptosis, decreased lymphocyte production in the bone marrow, or reduced lymphocyte proliferation in secondary lymphoid tissue [21].

Since PCV2 encodes for only two major proteins, it is thought that the virus relies on its host cell for protein expression and for replication. The virus requires an actively replicating cell, specifically in the S-phase [47], for DNA replication via a DNA polymerase. PCV2 replication is speculated to involve a rolling-circle method [48], [49]. It was determined in porcine kidney (PK-15) cells that the first detectable protein produced post-infection is the Cap protein, which was localized in the perinuclear area of the cell [50]. At 12 hours post inoculation, both Cap and Rep were detectable in the nucleus. By 36 hours post inoculation, the viral titer had stabilized indicating that the virus had completed replication [50]. There is currently no information on how the capsid is made, how the virus assembles or how it is released from infected cells.

1.7 Viral transmission

PCV2 can be transmitted in several ways. The main route is by oro-nasal contact with infected feces [11], contact with infected urine, or directly with infected pigs [51], [52]. PCV2 is shed in respiratory secretions, oral secretions, urinary secretions, and feces in both clinically-affected as well as in infected but apparently healthy pigs. Clinically affected pigs shed virus in higher quantity compared to infected but clinically healthy pigs. Pigs showing clinical signs shed virus in higher amounts [53]. PCV2 can also be transmitted vertically (from the sow to the piglets) through the placenta causing persistently-infected piglets at birth, but this method of transmission appears to be rare [54], [55], [56], [57]. PCV2 has also been shown to be shed in colostrum [58], but whether this can result in an infection is still being investigated. Recent work has shown that PCV2-infected pork products (lymphoid tissue, skeletal muscle, and bone marrow), when fed to naive piglets for 3 days, resulted in viremia and seroconverion to PCV2 in all of the piglets [59].

PCV2 is also shed in semen and in experimental studies seminal virus shedding was detected as early as 5 days post inoculation [60], [61], [62]. Shedding in naturally infected boars appears to be low and sporadic [60]. The greatest amount of virus appears in the seminal fluid and non-sperm fraction [63]. Boars that are persistently infected may continue to shed the virus in semen and semen samples were found to be positive in boars up to 71 weeks of age. Samples collected from boars ranging from 71 to 149 weeks of age were not found to shed virus in semen [60]. The virus did not appear to affect the percentages of live and morphologically normal sperm [60]. Recent evidence has shown

that PCV2 virus present in the semen is infectious when injected intraperitoneally into pigs, but failed to seroconvert gilts that were artificially inseminated. In addition, all pigs born of the gilts were negative for PCV2 antibodies [64], [65].

The incubation period in experimentally infected pigs ranges from 2 to 4 weeks [52], [66], [67], [68]. Once inside the host, PCV2 first infects the tonsils and lymph nodes of the head and begins replicating [69]. PCV2 also infects B-cells [70] which likely causes dissemination throughout the body via the lymphatic system. PCV2 has been detected in the spleen, Peyer's patches, and many lymph nodes [71]. PCV2 then starts replicating in T-cells and peripheral blood mononuclear cells [70], [72]. Viremia in pigs is detectable between 7 and 14 days post inoculation. PCV2 has the ability to cause a prolonged infection, with viral DNA detectable in pigs up to 125 days post inoculation in experimental infections [51], [52], [66], [67], [68], [73], [74], [75].

1.8 Host Immunity

Since most breeding age sows are seropositive for PCV2, most piglets are born with maternal antibodies against PCV2 [76]. In weaned piglets, the mean half life of antibodies is 19 days. Antibody levels will wane at 4-6 weeks in pigs with initially low levels of antibody, at 6-10 weeks with moderate antibody levels and by 8.5-13.5 weeks in pigs with high antibody levels [18]. Piglets do not typically demonstrate clinical signs of disease prior to 4 weeks of age suggesting that maternally-derived antibodies are protective [76], [77], [78], [79]. Experimental studies found that maternal antibody protection is dependent on the level of maternal antibodies present. High levels of maternal antibodies are more protective than low levels, but do not completely prevent

infection, while low levels of antibodies did not provide any protection against infection [76].

Two to three month-old pigs are capable of producing an antibody response to PCV2 infection, but this response is not completely protective as these pigs may still develop viremia [80], [81], [82]. Experimental infections show that pigs seroconvert between 14 and 28 days post infection (DPI) [66], [67], [74], [83]. By 10 days post infection (DPI), pigs can develop neutralizing antibodies that increase in titer up to 21 DPI [84]. Neutralizing antibodies were detected in naturally-infected Belgian pigs by 10 weeks of age, and in Danish pigs at 3 weeks of age. Pigs that develop PCVAD have low or undetectable levels of neutralizing antibodies [84].

PCV2 pathogenesis appears to be related to the immunomodulatory effects of the virus. PCV2 infection results in a decreased expression of B-cell growth factor IL-4 and the cytotoxic T-cell and macrophage activating cytokine IL-2 [85]. This results in a decreased proliferation of lymphocytes and the interferon antiviral response [86] while causing an increase in expression of pro-inflammatory cytokines IL-1B and IL-8 [85].

PCV is believed to be a species-specific virus, however antibodies to PCV1 have been detected in mice, cattle, and humans [87], but currently PCV2 is not considered to be a zoonotic disease. However, with the advent of xenotransplantation using porcine organs, the risk of implanting PCV2 infected organs into immunocompromised xenograft recipients should be investigated.

1.9 Factors that modulate diseases caused by PCV2

PCV2 infection is characterized by having a high prevalence of infection but low morbidity, and thus not all animals infected with PCV2 will develop clinical signs of PCVAD [88], [89], [90] Seroprevalence in commercial herds in some countries is near 100% [90], [91]. While most pigs in a herd will become viremic, only 5-30% of susceptible pigs will show clinical signs of PCV2 [88], [90]. There are four main factors essential in the expression of PCV2-related diseases: viral effects, host effects, and the effects of co-infection and immunomodulation [21].

1.9.1 Viral factors

Although PCV2 is capable of causing several distinct disease syndromes, there are no significant differences in the virus genomes recovered from the different syndromes. Sequence analysis of PCV2 from PCVAD-affected pigs, and pigs with clinically unapparent infection showed 95.6% to 100% sequence homology and no distinct patterns of sequence variations were evident between the two groups. This has lead to the belief that there are other factors affecting the expression of disease [92]. It has been demonstrated that PCV2 isolated from pigs without disease can cause PCVAD under experimental conditions [93], [94].

It was shown that two amino acid mutations in the PCV2 genome significantly altered the gross and histopathological lesions seen in pigs, indicating that only minor alterations in the viral genome are required to alter the function of the virus [95]. In the Canadian outbreak of 2004, a change in virus type was demonstrated which caused a much more severe disease characterized by pulmonary edema, granulomatous enteritis,

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more severe lymphoid depletion and lymphoid necrosis [96]. Subsequent reports of the introduction of PCV2b into the United States were associated with severe outbreaks in Kansas, Iowa and North Carolina in 2006 [31]. It is unknown if the increased prevalence of PCV2b is associated with a change in virulence, new introduction to the area, or other factors that allowed an increase in replication of this viral type.

1.9.2 Host factors

All breeds of pigs appear to be susceptible to infection, and clinical disease has been observed in many purebred and crossbred pigs [P.G. Halbur, unpublished data]. However, studies have shown differences in susceptibility in different breeds of pigs [97], [98]. Differences in the type of adaptive immune response against PCV2 in different pigs may explain the host variation in the outcome of infection [99]. One study found significant differences in the replication patterns of PCV2 in alveolar macrophages from different conventionally crossbred pigs [50].

1.9.3 Co-infection

While PCV2 is required to cause the characteristic lymphoid depletion of PCVAD, many strains likely require a co-factor to cause the full spectrum of clinical signs associated with PCVAD. Co-infection with several other viral and bacterial pathogens has been shown to cause an increase in incidence and a markedly more severe clinical course of disease. The agent implicated as creating the greatest risk is porcine reproductive and respiratory syndrome virus (PRRSV) [100]. Other agents include porcine parvovirus (PPV) [66], [73], [101], [102], [103], *Mycoplasma hyopneumoniae*

[104], and very recently torque teno virus (TTV), which singly is not associated with disease but present in many pig populations [105], [106]. A retrospective analysis of the number of PCVAD cases in which there were co-infections was performed by the Iowa State Veterinary Diagnostic Laboratory. The results showed that more than 98% of pigs had co-infections. Specifically, 52% were co-infected with PRRSV, 36% with *Mycoplasma hyopneumoniae* 15% with porcine parvovirus, 14% with bacterial septicemia, 7.6% with bacterial pneumonia, and 5.4 % with swine influenza virus. A single PCV2 infection occurred in only 1.9% of the cases [107].

1.9.4 Immunomodulation

Part of the pathogenesis of co-infection causing more severe disease may be associated with immunostimulation prior to PCV2 infection. One study showed that pigs which were immunostimulated with keyhole limpet hemocyanin developed clinical PCVAD when infected with PCV2 [83]. There is also mounting evidence that common adjuvanted vaccination regimens may actually enhance the development of PCVAD [108], [109], [110]. In pigs vaccinated with the same antigen, but different adjuvants, the oil-in-water adjuvant was shown to cause a longer length of viremia, increased amounts of PCV2 in serum and tissue, and more severe lymphoid depletion when compared to pigs vaccinated with aqueous and aluminum hydroxide products [111]. Similarly, vaccination with *Mycoplasma hyopneumoniae* or *Actinobaccilus pleuropneumoniae* vaccines followed by immediate infection with PCV2 in specific-pathogen-free (SPF) pigs caused a significant increase in viremia duration and more severe histopathological lesions than in non-vaccinated pigs [110].

The effects of immunosuppression on disease caused by PCV2 have also been studied. Infection of pigs with PCV2 after injection with cyclosporine caused an increase in PCV2 replication, and a higher titer of virus compared to controls, but the pigs did not develop clinical PCVAD [112]. In another study, pigs treated with dexamethasone prior to PCV2 infection developed a granulomatous lymphadenitis that was not observed in pigs inoculated with PCV2 alone [113]. In addition, a series of studies indicate that cell-mediated immunity plays an important role in protection [43], [76], [99], [114]. A proportion of pigs vaccinated with a live PCV1-2 chimeric vaccine developed only low levels of antibody and yet the vaccinated pigs were fully protected against subsequent challenges with PCV2 [114], [115].

1.10 Porcine Circovirus Associated Disease (PCVAD)

PCVAD recently replaced the older name of PMWS. The name PCVAD was adopted to be inclusive of all the recognized syndromes associated with PCV2 infection [116]. According to the American Association of Swine Veterinarians (AASV) PCVAD case definition (posted October 2006 at www.aasp.org/aasv/position-PCVAD.htm), PCVAD can be subclinical or include one or more clinical manifestations including multisystemic disease with weight loss and high mortality, respiratory disease, porcine dermatologic and nephropathy syndrome, enteric signs including diarrhea, and reproductive disorders on an individual or herd basis. Distinguishing the different forms of PCVAD can be accomplished by observation of gross or histopathologic characteristic lesions in the intestines, lungs and lymphoid tissue [21].

1.11 Syndromes

1.11.1 Porcine Multisystemic Wasting Syndrome (PMWS)

The most significant manifestation of PCVAD is the multisystemic syndrome. This syndrome has been recognized in wild boars, but the source of the infection is believed to be the domestic pig [117]. This disease affects pigs between 7 and 16 weeks old in the United States and 5 to 12 weeks old in Europe [5], [118], [119]. This age difference is most likely related to variation in management practices and vaccination timing between producers in the US and Europe [110]. Morbidity is associated with the development of viremia and lymphopenia in piglets followed by the clinical manifestations of disease. Mortality is usually around 10% [5], [9], [118], (range 4-20%) [118], but can reach 50% [5], [9]. Since the clinical course of wasting and decreased economic efficiency can be prolonged, 70-80% of pigs that develop PCVAD are subsequently euthanized [118].

Clinical signs of PCVAD include wasting with progressive weight loss, lethargy, dark colored diarrhea, lymphadenopathy, and paleness or jaundice. The main characteristic histopathological lesions are lymphoid depletion with histiocyte (any macrophage found in connective tissue) replacement in lymphoid tissues, and intracytoplasmic inclusion bodies [4], [5], [9], [11], [69], [120]. Early signs often go unnoticed or are misdiagnosed; these include reduced weight gain, ill-thrift, pale skin and rough hair coat. Later signs include dyspnea, tachypnea, anemia, diarrhea and jaundice [9]. Pigs may also have coughing and gastric ulceration, which most likely contributes to the anemia. On necropsy, the lungs will fail to collapse and are mottled, tan-colored, and in chronic cases the kidneys may have white streaks or spots [21]. They also have

enlargement of the superficial inguinal, submandibular, mesenteric and mediastinal lymph nodes [69]. Granulomatous lesions may also be found in the lungs, liver, kidney, heart and intestines [21].

A scoring system has been developed which estimates the severity of disease based on the extent of lymphatic tissue involvement. The seven lymphoid tissues that are evaluated for the purpose of scoring include the tracheobronchial lymph nodes, the mesenteric lymph node, the mediastinal lymph nodes, the superficial inguinal lymph nodes, the external iliac lymph nodes, the tonsils and the spleen. This system accounts for the severity of lesions, the amount of PCV2 antigen and the distribution of the lesions. Scores are assigned and range from 0-9 [104]. Although this system is useful for classifying the severity of disease, it is impractical for field necropsies.

1.11.2 Subclinical PCV2 infection

PCV2 infection can be limited to 1 or 2 lymph nodes in the absence of evidence of clinical disease [104], [121]. However, the presence of PCV2 may be associated with a decrease in vaccine efficacy [122] and healthy pigs may still exhibit a necrotizing lymphadenitis [121], [123]. The significance of this finding to the pig is unknown, but may cause the carcass to be condemned at slaughter [21].

1.11.3 PCV2 associated enteritis

This syndrome affects piglets from 8-16 weeks old and resembles chronic ileitis associated with *Lawsonia intracellularis* infection. Affected piglets have diarrhea,

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unthriftiness, retarded growth, and increased mortality. Histopathological lesions include a granulomatous enteritis and characteristic PCV2 lesions in Peyer's patches but not in other lymphoid tissues. At necropsy, mesenteric lymph nodes are enlarged and the intestinal mucosa is grossly thickened. Histopathology is able to easily distinguish between *Lawsonia* versus PCV2 infections [124].

1.11.4 PCV2 associated pneumonia

This syndrome may play a role in porcine respiratory disease complex [125], [126]. It affects pigs from 8-26 weeks old and is associated with multiple pathogens. The clinical signs include decreased rate of growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea. This can be very similar to systemic infection and there is some overlap of the syndromes. The histopathological lesions include a granulomatous bronchointerstitial pneumonia with mild to severe necrotizing and ulcerative bronchiolitis and bronchiolar fibrosis. Differentials for the bronchiolitis lesions may include swine influenza or porcine respiratory coronavirus infections [21].

1.11.5 PCV2 associated reproductive failure

This syndrome was first reported in Canada in 1999 [127] and typically affects gilts and start up operations [128]. The clinical signs include increased abortion, still births, fetal mummies and pre-weaning mortalities. The histopathological lesions include a non-suppurative to necrotizing or fibrosing myocarditis in still born and neonatal pigs [128]. The time of infection determines the clinical course of the disease. Fetuses inoculated at 57 days of gestation had higher viral replication than those infected later in

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gestation and when killed at 21 days post inoculation had edema, enlarged livers and congestion. Fetuses inoculated at 75 and 92 days of gestation failed to produce similar lesions or viral loads [129]. Late term infections at 86, 92 and 93 days of gestation caused an increase in reproductive abnormalities including still birth, fetal mummies and weak piglets [130]. However, data from field cases indicates that most breeding herds appear to be immune to this disease [21].

1.11.6 Porcine Dermatitis and Nephropathy Syndrome (PDNS)

This syndrome was first described in the UK in 1993 [131] and was associated with PCV2 in the year 2000 [132]. This disease is often fatal within 3 days of development and mostly affects grower pigs, but can affect pigs as young as 5 weeks old. Clinical signs include an acute onset of fever, lethargy, and raised purple skin lesions progressing to multifocal red-purple scabs with black centers being most prominent on the rear legs. At necropsy the kidneys are enlarged, tan and waxy in appearance with petechial hemorrhages. Histopathologically, there is a systemic vasculitis with dermal and epidermal necrosis and necrotizing and fibrinous glomerulonephritis appearing similar to a type 3 hypersensitivity reaction with deposition of antigen-immune complexes in the vascular and glomerular capillary walls [21]. The development of disease is aided by co-infection with PRRSV [133], [134], *Pasteurella multocida*, *Streptococcus suis* types 1 and 2, among others [135], [136]. Recently, PDNS was experimentally reproduced with PRRSV and TTV in PCV2 free pigs [137]. Therefore, PDNS is not always associated with PCV2.

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1.11.7 PCV2 associated neuropathy

In 2001, PCV2 was associated with pigs born with congenital tremors and a non-supperative menigoencaphalitis located in the brain [41], [57], [138]. More recent reports have associated PCV2 infection with cerebellar lymphohistiocytic vasculitis combined with hemorrhages or with lymphohistiocytic meningitis. PCV2 antigen was found with immunohistochemistry in the cytoplasm and nuclei from intralesional perivascular machrophages and endothelial-like cells in the brain tissue [139]. In addition, naturally occurring neurologic disease characterized by opisthotonus, nystagmus, and convulsions was associated with PCV2 infection in pigs ranging from 6-8 weeks old in which cerebellar vasculitis was also present [140]. The role of PCV2 in the development of this disease is still under investigation, but may indicate a new spontaneously occurring type of PCV2 disease.

1.12 Diagnosis

The diagnosis of PCVAD is based on clinical signs and demonstration of PCV2 antigen in more than 1 lymphoid tissue, or 1 lymphoid tissue and 1 other organ system such as the lungs, liver, kidney or intestine, or in 2 organ systems. If antigen is found only in 1 organ system, then the disease is categorized based on that organ system. If only limited PCV2 antigen is found but there are severe lesions, it is classified as chronic severe PCVAD21. Scoring of lesions and the amount of antigen in tissues allows for staging of infection [21]. Diagnosis can be tentatively made based on clinical signs. The prevalence of clinical signs was reported in several farms experiencing PCVAD disease (Table 2).

Detection of PCV2 antigen or nucleic acid is considered the gold standard for the diagnosis of PCVAD. The best tests for this are polymerase chain reaction (PCR), *in situ* hybridization (ISH) and immunohistochemistry (IHC) [21]. There is currently no information on sensitivity and specificity of these tests, but IHC gives more intense staining and is considered more sensitive, but less specific than ISH. IHC is also cheaper to run and has a faster turn around time. However, many labs do not offer IHC, since one of the required reagents is anti-PCV2 antiserum. Although a monoclonal anti-PCV2 is commercially available, definitive diagnosis can still be difficult with that product. The best way to diagnose PCVAD is the identification of the characteristic lesions of the disease. Microscopic lesions associated with PCVAD include syncytial cells in lymph nodes, Peyer's patches, and the lamina propria of intestinal villa. In addition, macrophages have sharply demarcated, spherical, basophilic cytoplasmic inclusion bodies [69].

Serology can also be performed and is a convenient method for detecting exposure to PCV2 for large numbers of pigs. However it must be remembered that many clinically healthy pigs are seropositive. Other tests that have been developed include immunofluorescence assay, IgM immunoperoxidase monolayer assay (IPMA), enzyme linked immunosorbant assay (ELISA), virus isolation, electron microscopy and serum virus neutralization assays [7], [13], [21], [37], [74], [87], [99], [101], [141], [142], [143], [144], [145], [146]. There is currently no field test for the diagnosis of PCVAD.

1.13 Classification and Management of Herd Outbreaks

No specific treatment is available for diseases associated with PCV2 infection. In general, treatment of individually affected pigs is supportive only and will vary greatly depending upon the clinical signs that the animal displays. Since many animals are co-infected, choosing appropriate therapy will also depend upon identification of the other agents infecting the animal. In addition, prognosis is dependent upon animal factors, such as age as well as the syndrome that the animal displays. There is currently no data on the existence of PCVAD in pet potbellied pigs, but some veterinarians are recommending PCV2 vaccination to their clients. During initial PCVAD outbreaks, pigs treated with antibiotics actually suffered a higher mortality rate than those not treated, but it is believed that this was more due to spreading the virus with common use needles than any effect by the antibiotics (Personal Communication: Dr. R.B. Baker, Iowa State University, Ames Iowa).

PVC2 related syndromes are of greatest economic concern when they occur in herd populations. To identify and manage outbreaks of PCV2 and PCVAD diseases within a herd, it is important to determine whether the disease is a significant herd problem or is only sporadically causing a herd problem. A definition has been developed to help with this determination. A significant herd problem has been defined as an increase in mortality of equal to or more than the mean of historic mortality levels plus 1.66 times the standard deviation. If there is no historical mortality data, a herd problem can be defined as an increase in mortality that exceeds the national or regional level by 50%. In other words, if 50% or more of the pigs from a representative sample are diagnosed with PCVAD and there has been a significant increase in mortality compared

to previous mortality data, it is considered a herd problem. However, if less than 50% of

the pigs are diagnosed with PCVAD, but there has still been an increase in mortality or if

more than 50% of the pigs are diagnosed with PCVAD but there has been no increase in

mortality, the outbreak may be considered sporadic and not a herd problem [147] (Table

3).

1.14 Prevention of PCV2 associated diseases

Prevention of PCVAD can be difficult. Disease outbreaks are reported to occur

on farms even with strict isolation practices. It has been shown that vaccination against

Mycoplasma hyopneumonia or *Actinobacillus pleuropneumoniae* 2-4 weeks in advance

of infection with PCV2 prevented any lesions associated with PCV2 infection [110], but

the practical issues of this method make it nearly impossible to accomplish. Porcine

parvovirus vaccination does not reduce the severity of PCVAD in co-infected pigs [103]

but a combined PPV and swine erysipelas vaccine appeared to protect against PCV2-

induced reproductive failure [148].

Treatment of bacterial infections and prevention of co-factor associated diseases

is also a good practice in preventing PCV2 diseases. Treating *M. hyopneumonia* with

chlorotetracyclines was highly effective [149]. Bleach is an effective chemical in killing

PCV2, but has unknown field efficacy. The protocol utilized at Iowa State University to

disinfect pens is included in table 4 [21].

Good housing management is critical in disease prevention. It has been shown

that reducing stress, paying attention to proper hygiene, preventing mixing of ages and

utilizing all in/all out practices are effective in controlling disease. Other options include

immunized serum therapy, which has practical limitations, and depopulation, which has been shown to be ineffective since the virus is very resistant in the environment [150]. Whether PCV2 can be found in insects or wild animals which could possibly transmit disease to pigs is not known. However, since circoviruses are highly species-specific, it is unlikely that these animals, excluding feral boars, would pose a threat of PCV2 transmission to domestic herds [151].

Risk factors for development of infection include PPV or PRRSV infection, large pen sizes versus small pen sizes for weaning piglets, increased levels of cross fostering [152] and vaccination against PRRSV [153]. As such, reduction of these risk factors on farms may be helpful in controlling PCVAD. A study in Canada showed a strong association of increased mortality with *M. hyopneumoniae* infection, PRRSV, diarrhea caused by *Escherichia coli* K88, close proximity to other herds, multiple suppliers, large within-group range in age of pigs and not using spray-dried plasma in first nursery rations [154]. Factors that decreased the risk included long empty times between pig groups, regular treatment of external parasites, pen versus crate gestation, internal versus external gilt replacement [152], and vaccination against atrophic rhinitis [153].

1.15 Vaccine development and vaccination

Initial attempts at vaccine development included using a killed porcine parvovirus vaccine. Since pigs are often co-infected with PPV and PCV2, it was hoped that vaccination at an early age would prevent PCVAD. This approach empirically appeared promising in the field, but a benefit was not confirmed when the vaccine was tested under more controlled conditions [103], [155].

One of the first PCV2 vaccines on the market was Merial's CIRCOVAC®, an inactivated PCV2 vaccine with oil adjuvant for use in breeding age animals [21]. This vaccine was most extensively used in Europe but it was also available in Canada. The vaccine was successful in reducing PCV2 circulation and shedding in the first weeks of life of the piglets born to the vaccinated sows [156]. This vaccine is designed to be given as 2 injections intramuscularly 3-4 weeks apart and completed at least 2 weeks before breeding and once at each subsequent gestation [21].

Another vaccine available in the United States is the Boehringer Ingelheim's Ingelvac CircoFLEX® vaccine, a capsid based subunit vaccine expressed in inactivated baculovirus. This vaccine demonstrated significant decreases in mortality in vaccinated pigs versus unvaccinated pigs on 4 different Canadian finishing sites [157]. This vaccine is to be given in a single dose intramuscularly to piglets greater than 3 weeks of age [21]. A field trial for this vaccine recently was reported in the UK in which 3 week old pigs were vaccinated. Mortality caused by PCVAD was reduced from 14.3% to 4.6% [158].

A third vaccine which is produced by Intervet is also a capsid based subunit vaccine. This vaccine, expressed in a baculovirus, is marketed under the name Circumvent® PCV in the United States and Canada and Porcilis® PCV in Europe and Asia. It is designed for vaccination of piglets 3 weeks and older. Circumvent® PCV is given in 2 doses 3 weeks apart at 3 and 6 weeks of age given intramuscularly [21]. Porcilis® PCV only requires one dose. Studies including 35,000 pigs on 21 different farms showed that mortality of vaccinated pigs was reduced by 77.5% when compared with unvaccinated pigs [159].

The first USDA-fully licensed PCV2 vaccine was licensed for use in pigs 4 weeks of age or older. This genetically engineered chimeric vaccine was created by inserting the immunogenic capsid protein of PCV2 into the genetic backbone of the non-pathogenic PCV1 [95], [114], [115], [160]. This chimeric vaccine was shown to be attenuated in pigs and was able to prevent the viremia and lymphopenia associated with PCV2 morbidity [114], [115]. This vaccine was named PCV1-2 to indicate its chimeric nature, and was released in July 2006 in a killed form labeled as Suvaxyn® PCV2 One dose™ (Fort Dodge and Wyeth Animal Health). The vaccine is given as a single dose intramuscularly.

Field studies on Suvaxyn® PCV2 One dose™ indicate that it is very effective and safe. The vaccine is able to decrease the mortality from 8-10% in non-vaccinated pigs to 1.0-2.0% in vaccinated pigs. Safety studies have been completed on over 1,000 pigs in four different locations. No adverse reactions were recorded in any of the vaccinated pigs (Table 5). In addition, experiments have proven that the PCV1-2 vaccine is able to break through maternal antibodies to provide protection in piglets against PCV2 infection. This allows piglets to be vaccinated before maternal antibodies wane and before the typical age of infection with PCV2 [161]. The live version of the chimeric PCV1-2 vaccine has also been demonstrated to be genetically stable in vaccinated pigs [162], and thus could serve as a good candidate for a live vaccine against PCV2.

1.16 Conclusions

Since its initial discovery in 1991, PCV2 and PCVAD have had a significant and adverse impact on the economy of the swine industry. There are currently seven

recognized syndromes related to PCV2 infections. Many of these syndromes are a result of co-infection with PCV2 virus and other agents like *Mycoplasma* and PRRSV. Diagnosis of PCV can occasionally be difficult due to nondescript clinical signs but diagnostic lab tests are available. The current knowledge and research into vaccines is providing relief for the swine producer from the heavy losses associated with the disease. Key points in reducing loses are centered on proper vaccination and management. The reduction in swine loses already being witnessed after the introduction of vaccines in the world market has been significant, and further research to provide even better vaccines will likely continue to reduce economic loss in the world swine market.

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1.18 Tables

Table 1: Prevalence of PCVAD by type of operation, farm size, and year

Year	Age Group	Farm Size			
		<2000	2000-10,000	>10,000	Overall
2000	Nursery	4.4	10.4	20.9	**5.7%**
	Grower and Finisher	2.3	8.8	12.4	**3.6%**
2006			Farm Size		
		<2000	2000-5000	>5000	
	Nursery	21.5	12.5	39.6	**22.3%**
	Grower and Finisher	25.0	35.4	59.9	**31.1%**
PDNS	Nursery	3.3	0.0	3.4	**2.9%**
	Grower and Finisher	1.6	10.4	23.9	**6.0%**

Table 2: Clinical signs of PCVAD

Clinical sign	Percentage of sites that reported the clinical sign in their PCVAD positive pigs
Wasting	98.1
Diarrhea	77.2
Dyspnea	75.1
Lymphadenopathy	44.8
CNS signs	39.6
Jaundice	37.1
Off-feed	90.4
Death	96.8

Table 3: Definition of Significant and Sporadic Herd Outbreaks

Significant	• Increase in mortality $\geq$ mean historical mortality for that herd plus 1.66 x standard deviation • In the absence of historical data, an increase in mortality that exceeds the national or regional levels by 50%
Sporadic	• Less than 50% of sick pigs have been diagnosed with PCVAD from submitted samples but there is still an increase in mortality • More than 50% of pigs are diagnosed with PCVAD from submitted samples but there has been no increase in mortality

Table 4: Iowa State University Disinfection Protocol

- Apply degreaser detergent with foamer at a 1:64 dilution. (Iowa uses Grease Free PV, but any product should work the same)
- Leave for 10 minutes
- Remove with pressure washer using hot water
- Decontaminate with Virkon S[1] at 1:30 dilution
- Leave for 10 minutes
- Rinse with hot water
- Prior to occupancy, fog with Clindox-S[2] at 1:5:1 dilution and let dry
- Rinse with water 6-12 hours after fogging and allow to dry

  1   Antec International, Sudbury, Suffolk UK
  2   US Pharmacal Com LLC, Erie, CO

Table 5: Summary of available PCV2 vaccines

Vaccine Name	Meriel Circovac®	Boehringer Ingelheim Ingelvec CircoFLEX®	Intervet/SP Circumvent® PCV	Fort Dodge Suvaxyn® PCV2 One dose™
Type	Inactivated PCV2	PCV2 ORF2 in baculovirus	PCV2 ORF2 in baculovirus	Inactivated PCV1-2 chimera
Administration	2ml IM	1ml IM	2ml IM	2ml IM
Advantages	Breeding age animals	Single dose No oil adjuvant		No oil adjuvant Single dose
Disadvantages	2+ doses per gestation Needs reconstitution Mineral oil adjuvant		2 doses Local reaction may occur	

1.19 Figures

Figure 1: Genomic Organization of PCV1 and PCV2
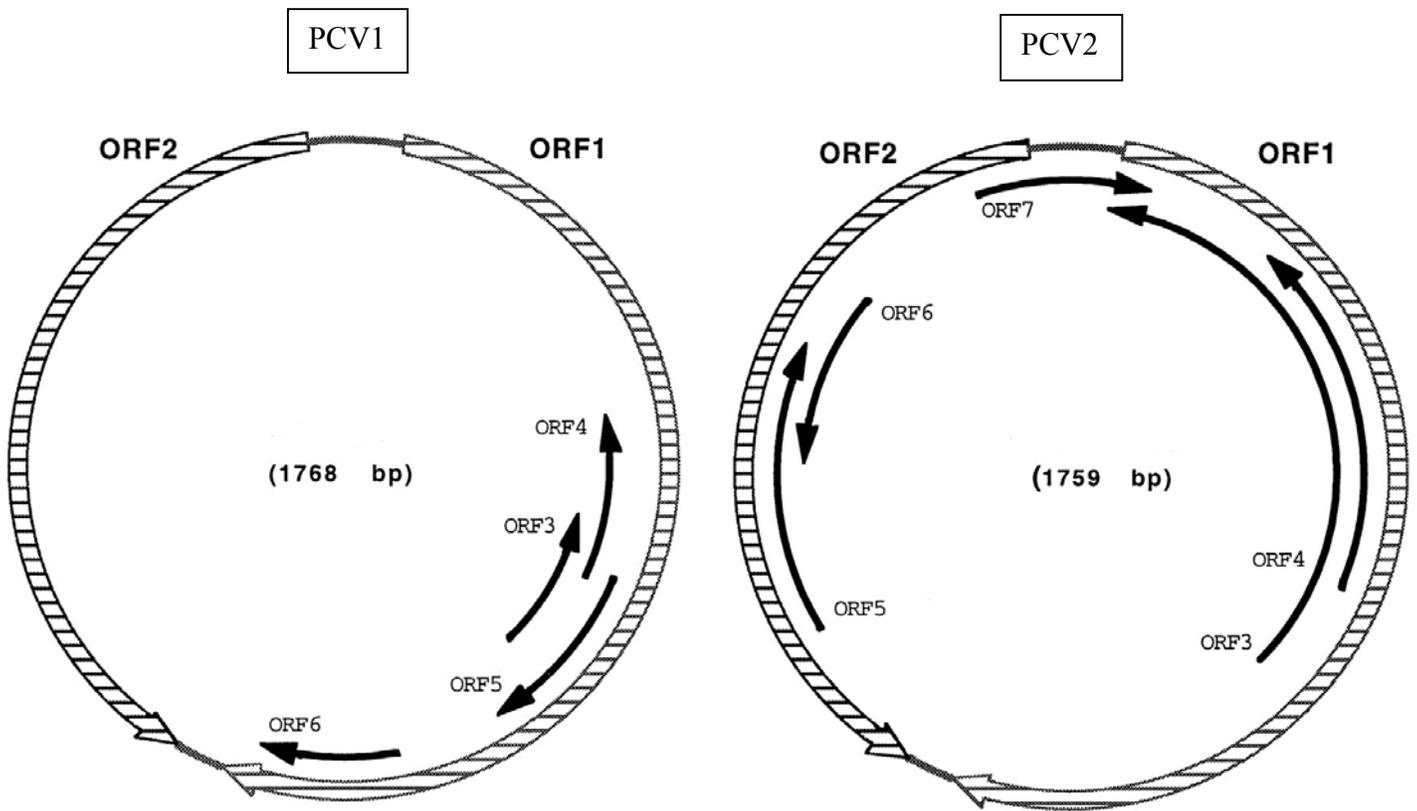
Chapter 2

A genetically-engineered chimeric vaccine against porcine circovirus type 2 (PCV2) is genetically stable *in vitro* and *in vivo*

J. Gillespie, N.M. Juhan, J. DiCristina, K.F. Key, S. Ramamoorthy, and X.J. Meng

Abstract

A vaccine against porcine circovirus type 2 (PCV2), designated PCV1-2 chimera, was recently developed by replacing the capsid gene of the non-pathogenic PCV1 with that of PCV2. The PCV1-2 chimera virus is attenuated in pigs but induces protective immunity against PCV2. In this study, the genetic stability of the PCV1-2 chimera was evaluated for its potential use as a live vaccine. The PCV1-2 chimera virus was serially passaged 11 times in PK-15 cells and 3 times in pigs. The PCV1-2 chimera virus used in this study contained a tracking marker mutation in the capsid gene (F to V at amino acid position 79). Sequence analyses of the PCV1-2 chimera virus after 11 serial passages in PK-15 cells did not reveal any sequence change including the marker mutation. Similarly, there is no change in the genomic sequence of the PCV1-2 chimera virus recovered from pigs during 3 serial *in vivo* passages. Under *in vivo* selection pressure, however, the introduced tracking marker mutation in the PCV1-2 chimera quickly mutated (V79F) and restored to its original sequence after one passage in pigs, and remained stable in subsequent 2 passages in pigs. The results indicate that the PCV1-2 chimeric virus is genetically stable, and thus should be a good vaccine candidate.

Key words: Porcine circovirus type 2 (PCV2); Porcine circovirus-associated disease (PCVAD); vaccine; genetic stability.

Abbreviated title: Genetic stability of a PCV2 vaccine

2.1 Introduction

Porcine circoviruses (PCV) are minute viruses that belong to the family *Circoviridae* [4], [10], [30], [33]. PCV has a small, non-enveloped icosahedral structure [34] with a single-stranded, circular DNA genome of approximately 1.76 kb in size [11], [24]. The viral genome consists of 2 major open reading frames (ORFs): ORF1 encodes for viral replication proteins, and ORF2 encodes for the immunogenic capsid protein [6], [7], [21], [22], [24].

There exist two, phenotypically distinct but genetically related, types of PCV [11], [18], [28]. PCV type 1 (PCV1) was originally identified as a persistent cell culture contaminant of the porcine kidney cell line PK-15 (ATCC CCL-33) [34]. Subsequent studies showed that PCV1 does not cause disease and is thus non-pathogenic in swine [1], [4], [35]. Porcine circovirus type 2 (PCV2) was first identified in association with a disease now known as Porcine Circovirus-Associated Disease (PCVAD) in weaning piglets in Canada in 1991 [2], [4], [10], [17]. Currently, PCVAD is an economically important global swine disease that affects pigs between 7 and 15 weeks of ages with a mortality rate of up to 50% [17], [19], [20], [29], [30], [32]. Clinical signs of PCVAD include wasting with progressive weight loss, lethargy, diarrhea, lymphadenopathy, and jaundice. The characteristic histopathologic lesions are lymphoid depletion with histiocytic replacement in the lymphoid tissues and organs [1], [4], [17], [19], [32], [33]. PCV2 has now been identified from pigs in almost all of the major swine producing countries worldwide [3], [4], [9], [10], [11], [30], [33]. Despite the distinct pathogenic

property between PCV1 and PCV2, they share approximately 76% nucleotide sequence identity across the entire genome [6], [7], [11].

Several killed or recombinant vaccines are currently available [10], [16], [19], [30], [31], [33] but there is no live-attenuated vaccine against PCV2. Recently, a chimeric PCV1-2 virus was developed by replacing the immunogenic ORF2 capsid gene of PCV1 with that of PCV2 in the genomic backbone of the non-pathogenic PCV1 [12], [13], [14], [15]. Subsequent studies demonstrated that the PCV1-2 chimera virus is attenuated in pigs [13] and induce protective immunity against PCV2 infection and PCVAD in pigs [14]. The PCV1-2 chimera vaccine, Suvaxyn® PCV2 One Dose™, is currently being marketed in a killed form. Although the killed PCV1-2 chimera vaccine is effective, it is still advantageous to develop a live version of the PCV1-2 chimera vaccine against PCV2 and PCVAD. It has been demonstrated that some pigs vaccinated with the live PCV1-2 chimera vaccine virus developed only low level of antibody response and yet the vaccinated pigs were fully protected against subsequent challenges with PCV2 [13], [14], indicating that cell-mediated immunity may also play an important role in protection [14], [23], [25], [26]. Although the chimeric PCV1-2 vaccine virus is attenuated in pigs [13], [14], for its potential use as a live vaccine, it is essential to assess its genetic stability in pigs.

2.2 Materials and Methods

2.2.1 Cells, infectious DNA clone, and PCV1-2 chimera vaccine virus

A subclone of the PK-15 cell line that is free of PCV1 contamination was produced previously by end-point dilution of the PK-15 cells (ATCC CCL-33) [12], [13].

The chimeric PCV1-2 infectious DNA clone was generated previously [13], [14] by swapping the ORF2 capsid genes between the non-pathogenic PCV1 strain [11] and the pathogenic PCV2 strain ISU40895 [11] using the non-pathogenic PCV1 as the genomic backbone. The PCV1-2 chimera vaccine virus stock used in the study was produced by transfection of PK-15 cells with the chimeric PCV1-2 infectious DNA clone as described previously [13], [14], [15]. The PCV1-2 chimera vaccine virus stock was titrated by an immunofluroescent assay (IFA) as described previously [13], [14], [15], [21].

After determining the complete sequences of the PCV1-2 chimera, we found an unintentionally-introduced mutation, likely during PCR amplification and cloning steps, in the resulting PCV1-2 chimera. A single nucleotide change from a C to an A at nucleotide position 237 of the capsid gene resulted in a phenylalanine to a valine change at amino acid position 79 of the original PCV2 ORF2 capsid gene [11] in the PCV1-2 chimera infectious DNA clone and chimera virus stock. Since the F79V mutation did not affect the viability of the resulting PCV1-2 chimera virus, this F79V mutation was not changed back and instead was used as a marker mutation in this study to track the evolution of the PCV1-2chimera virus during serial *in vitro* and *in vivo* passages.

2.2.2 Serial passage of the PCV1-2 chimera vaccine virus in PK-15 cells

PK-15 cells were grown in a T-25 flask until they reached approximately 65% confluency. The cells were then infected with the PCV1-2 chimera vaccine virus. After incubation at 37°C for 1 hour, the inoculum was removed and 8 ml of minimal essentials media (MEM) containing 2% fetal bovine serum (FBS) and 2 X antibiotic-antimycotic was added to each flask. After incubation at 37°C for 3 days, the cells were split by

trypsinization into new flasks, and continued to incubate at 37°C for 3 days. The flask

was frozen and thawed 3 times to release the virus. This virus stock was used to infect a

new flask, and this process was repeated until 11 serial passages were reached.

Virus stocks collected from each serial passage were tested by an IFA to confirm

the presence of the PCV1-2 chimera vaccine virus in each passage. The IFA was

essentially carried out as described previously [12], [13], [14], [15]. Viral DNA was

extracted from virus stocks collected at passages #5 and #11 using a QIAamp DNA mini

kit (Qiagen, Inc., Valencia, CA) according to the protocol supplied by the manufacturer,

and stored at -80°C for further PCR amplification.

2.2.3 Serial passage of the PCV1-2 chimera vaccine virus in pigs

All animal experiments were carried out at the Swine Research Facility located at

the Center for Molecular Medicine and Infectious Diseases at Virginia Tech. The study

protocols were approved by Virginia Tech IACUC.

For *in vivo* passage #1, nine, approximately 7-week-old, specific-pathogen-free

(SPF) conventional pigs were divided into three groups of three pigs each (Figure 1).

The pigs were obtained from a high health breeding herd at Virginia Tech Swine

Research Center (Blacksburg, VA) and were free of common swine pathogens including

PCV1 and PCV2. Prior to the study, all piglets tested negative for PCV2 antibodies by an

ELISA [27]. The pigs in group 1 were each inoculated intramuscularly with 200 μg DNA

of the chimeric PCV1-2 infectious DNA clone. The pigs in group 2 were each inoculated

with $2 \times 10^{4.5}$ $TCID_{50}$ of the chimeric PCV1-2 vaccine virus (half intramuscularly, and

half intranasally). The pigs in group 3 were each inoculated with sterile PBS (1.5 ml

intramuscularly and 1.5 ml intranasally) and served as controls. Serum samples were collected at weekly intervals from all pigs.

One pig from each experimental group was necropsied at 14, 21 and 28 days post-inoculation (DPI), respectively, to collect various tissue samples including the lung, mesenteric lymph nodes, superficial inguinal lymph nodes, intestinal contents, thymus and tonsils. Tissue samples were each homogenized in cold sterile PBS to make a 10% (w/v) tissue homogenate. Approximately 200 µl of each tissue homogenate was used for DNA extraction, and PCR amplification of the chimeric PCV1-2 genome. The amplified PCR products covering the entire PCV1-2 genome were subsequently sequenced.

For *in vivo* passage #2, tissue samples of lungs, mesenteric lymph nodes, superficial inguinal lymph nodes and tonsils collected from the DPI 28 pigs of each experimental group in passage #1 that tested positive for chimeric PCV1-2 viral DNA by PCR were pooled and homogenized to produce a 10% (w/v) tissue homogenate in PBS buffer. Four ml of the 10% tissue homogenates were subsequently used to inoculate, 2 ml intramuscularly and 2 ml intranasally, three new pigs for the *in vivo* passage #2 experiment (Figure 1). The inoculation and sample collection in passage #2 are similar to those described in passages #1.

For *in vivo* passage #3, tissue samples of lungs, mesenteric lymph nodes, superficial inguinal lymph nodes and tonsils collected from the passage #2 pigs necropsied at DPI 28 were again pooled and homogenized to produce the inoculum for passage #3 (Figure 1). The inoculation and sample collection in passage #3 are similar to those described above in passage #2.

2.2.4 PCR amplification of the complete PCV1-2 chimera virus genome from in vitro and in vivo serial passages

Three sets of primers were designed based on the published sequence of the PCV1-2 chimera vaccine virus [13], [14]. The primers amplify 3 overlapping fragments of the entire PCV1-2 chimera viral genome. The first set of primers, PCV2.2A (5'-ACCTCTTATGGGGTTGCG-3') and PCV2.2B (5'- TTGTAGCCTCATCCAAAGC-3'), amplify a 686 bp region; the second set of primers, PCV2.3A (5'-ACCTGTTCTTGACTCCACC-3') and PCV2.3B (5'-AGGGACACCTACTGGAAGC-3'), amplify a 633 bp region; and the third set of primers, PCV2.4A (5'-CTCCATGATATCCATCCC-3') and PCV2.4B (5'-CAGCGTCAGTGAAAATGC-3'), amplify a 630 bp region.

DNA was extracted using a QIAamp DNA mini kit (Qiagen Inc., Valencia, CA) from virus stocks harvested at passages #5 and #11 during the *in vitro* serial passages as well as from serum samples and 10% tissue homogenates of various tissues collected from each of the 3 *in vivo* serial passages. The extracted DNA was amplified by PCR using Platinum PCR Supermix High Fidelity (Invitrogen Corp., Carlsbad, CA) with the three sets of primers described above. The PCR reaction consisted of 38 cycles of denaturation at 94°C for 30 seconds, annealing at 53.5°C for 30 seconds, and extension at 68°C for 1 minute, followed by a final extension at 68°C for 7 minutes.

2.2.5 Nucleotide sequencing and sequence analyses

The expected PCR products were separated by electrophoresis on a 1% agarose gel, and the desired bands were excised from the gels and purified with a QIAquick Gel

Extraction Kit (Qiagen Inc., Valencia, CA). Both strands of DNA were sequenced using the PCR primers with an ABI automated DNA Sequencer at Virginia Bioinformatics Institute (Blacksburg, VA). The sequences were compiled and analyzed by the MacVector computer program (Oxford Molecular Ltd., Beaverton, OR). The resulting full-length PCV1-2 chimera virus genomic sequences from *in vitro* serially-passaged viruses as well as from viruses recovered from pigs in each of the 3 *in vivo* passages were analyzed and compared to the original PCV1-2 chimera virus and to each other.

2.3 Results

2.3.1. The PCV1-2 chimera vaccine virus is genetically stable in vitro.

The PCV1-2 chimera vaccine virus was consistently detected by IFA in PK-15 cells from all eleven serial passages. The viruses harvested at passages #5 and #11 were completely sequenced for the entire genome. Sequence analyses revealed that the PCV1-2 chimera vaccine virus remained genetically stable during the 11 *in vitro* serial passages, since no change in the sequence of PCV1-2 chimera viral genome over 11 serial passages was observed: the sequences from passages #5 and #11 are identical to that of the original PCV1-2 chimera virus. The introduced marker mutation F79V remain genetically stable in the PCV1-2 chimera virus during the 11 serial passages in PK-15 cells.

2.3.2. Viremia in pigs inoculated with the PCV1-2 chimera virus and plasmid DNA of PCV1-2 infectious clone during serial in vivo passages is transient and variable

Detection of viremia was variable between DPI 7 and DPI 14 in both the chimeric PCV1-2 live virus- and infectious DNA clone-inoculated pigs in all 3 *in vivo* passages.

However, by DPI 21 in passage # 3, the detection of viremia was more consistent, with all the pigs viremic until necropsies at DPI 28 (Table 1).

2.3.3. The PCV1-2 chimera vaccine virus is genetically stable during 3 serial in vivo passages in pigs

The PCV1-2 chimera viruses recovered from the six tissues on DPI's 14, 21 and 28 in pigs inoculated with the PCV1-2 chimera live virus or with the plasmid DNA of PCV1-2 chimeric infectious clone during passages #1, #2 and #3 were sequenced for the complete genome (Table 2). Sequence analyses revealed that there was no change, except for the introduced marker mutation, in the genomic sequences of the recovered viruses during the 3 *in vivo* passages.

In pigs inoculated with the PCV1-2 chimera live virus, by DPI 28 the introduced marker mutation in the PCV1-2 chimera restored to its original ORF2 sequence ("V79F back-mutation", from V to F at amino acid position 79 of the capsid) present in the donor PCV2 strain ISU-40895 [11] from all the examined tissues except the lungs (Table 2). The sequences of the PCV1-2 chimera virus recovered from pigs necropsied on DPI 14 and DPI 28 of passage #2 pigs confirmed the presence of the V79F back-mutation in all tissues including lungs. The V79F back-mutation was still present in viruses recovered from all tissues (except tonsil) of pigs necropsied on DPI 28 in passage #3 (Table 2). However, no other mutation was detected from viruses recovered from pigs during the 3 *in vivo* serial passages.

In pigs that were inoculated with the plasmid DNA of the PCV1-2 chimeric infectious DNA clone, the V79F back-mutation was also identified in viruses recovered

from all examined tissues of pigs. However, the V79F back-mutation was not detected until DPI 28 in passage # 2 (Table 2). The back-mutation was not detected in passage #1 pigs, nor in passage # 2 pigs before DPI 28 (Table 2). The V79F back-mutation was present in viruses recovered from all tissues of the pig necropsied on DPI 28 in passage #3. Thus, in pigs inoculated with the plasmid DNA of the PCV1-2 chimeric infectious DNA clone, the back-mutation occurred between DPI 28 of passage #1 and DPI 28 of passage #2 (Table 2).

In an attempt to more precisely identify when the V79F back-mutation occurred in the PCV1-2 chimeric infectious DNA clone group, the PCV1-2 chimeric viral genomic DNA was amplified from weekly serum samples and sequenced. The virus sequence from a single serum sample collected on DPI 14 from a passage #2 pig (ID #52, Table 2) which was eventually necropsied on DPI 28 contained the V79F back-mutation, suggesting that the back-mutation likely occurred between DPI 28 of passage #1 and DPI 14 of passage #2.

2.4 Discussion

PCVAD is a devastating swine disease that causes severe economic losses to the global swine industry [4], [10], [18], [20], [30], [33]. Although the current killed and recombinant vaccines are effective against PCV2 [10], [16], [19], [30], [31], [33], a live-attenuated vaccine will be more potent and could reduce the cost associated with vaccination. Recently, a live vaccine against PCV2 was developed by replacing the immunogenic ORF2 capsid gene of PCV1 with that of the PCV2 in the genomic backbone of the non-pathogenic PCV1 [13], [14]. This chimeric virus, designated PCV1-

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2 chimera, is attenuated in pigs [13] and induces protective immunity against PCV2 infection and PCVAD in pigs [14]. The killed form of the vaccine based upon the PCV1-2 chimera virus, Suvaxyn® PCV2 One Dose™, is currently on the market. To ensure the safety for its use as a potential live vaccine, it is essential that the genetic stability of the PCV1-2 chimera vaccine virus be evaluated. Therefore, in this study we assessed the genetic stability of the PCV1-2 chimera vaccine virus by serially passaging the PCV1-2 chimera vaccine virus 11 times in PK-15 cells, and 3 consecutive times in pigs. The viruses recovered from both the *in vitro* serial passages and *in vivo* serial passages were completely sequenced and compared to the original PCV1-2 chimera vaccine virus to determine its genetic stability.

Studies of vaccine genetic stability are very important to ensure that attenuated-live virus vaccines are not prone to mutation or reversion which could cause disease. For examples, to evaluate the genetic stability of the CAM-70 measles vaccine strain, Borges et al [5] performed 10 serial passages of the seed lot vaccine virus FMS-7 in chicken embryo fibroblasts primary cultures. The nucleotide sequences of the virus harvested at passages #3 and #10 were determined, and found to be identical to the original vaccine strain, indicating that the CAM-70 measles vaccine is genetically stable [5]. In another study, the genetic stability of the three Sabin oral polio vaccine (OPV) virus strains were evaluated *in vitro* after 10 serial passages in two different cell cultures, VK cells and Vero cells [8]. The OPV vaccines produced on either VK or Vero cells were also given to 21 infants and the poliovirus excretion patterns and rate of reversion in these infants were analyzed. The authors found that there was no difference in the rates of reversion in

vaccinated infants, and that using the Vero cells as opposed to the VK cells for producing the vaccines does not negatively influence the genetic stability of the vaccine virus [8].

Compared to RNA viruses such as poliovirus [8] and measles virus [5] which are generally prone to mutations due to the lack of proof-reading ability of RNA polymerase, DNA viruses are genetically more stable. In the current study, after 11 serial passages of the PCV1-2 chimera vaccine virus in PK-15 cells, not a single nucleotide mutation was identified in the PCV1-2 chimera vaccine viruses harvested at passage #5 or #11. Even the introduced marker mutation (F79V) in the capsid gene of the PCV1-2 chimera did not change or restore to its original nucleotide after 11 serial passages in PK-15 cells. The results from this *in vitro* serial passage study indicated that the chimeric PCV1-2 vaccine virus is genetically stable in the absence of selection pressure in the *in vitro* PK-15 culture system. This finding is not surprising, since it has been demonstrated that wild-type PCV2 virus is genetically very stable during *in vitro* serial passages in PK-15 cells [15]. It was previously shown that only two amino acid mutations were identified after a total of 120 serial passages of the wild-type PCV2 virus in PK-15 cells [15].

After three serial passages in pigs, the PCV1-2 chimera vaccine viruses recovered from various tissues of inoculated pigs in each of the three passages were completely sequenced for its entire genome, with the exception of the back-mutation of the introduced marker, no other mutation in the entire PCV1-2 viral genome was identified in viruses recovered from pigs during the 3 serial passages. Compared to the pigs inoculated with the live PCV1-2 chimera vaccine virus, the V79F back-mutation in pigs inoculated with the plasmid DNA of PCV1-2 chimeric infectious clone did not occur until passage #2. This is not surprising, since we have previously demonstrated that, although the

plasmid DNA of the PCV1-2 chimeric infectious clone can induce active virus infection in pigs by intramuscular inoculation of plasmid DNA of the infectious clone, the infection generally appears late compared to the live virus-inoculated pigs since it takes time for the plasmid DNA of infectious clone to circularize and re-combine *in vivo* before initiating an active infection in pigs [12], [13], [14]. The amino acid position 79 fell within an immunoreactive region between amino acid residues 64 and 84 in the capsid protein [21]. Therefore, it is not surprising to see that, under selection pressure, this introduced marker mutation has the tendency to restore to its original nucleotide in infected pigs. Except for this back-mutation which was not unexpected, the PCV1-2 chimera virus was genetically stable in pigs during serial *in vivo* passages. Detection of the PCV1-2 chimeric viral DNA in serum samples during the serial passages was minimal, and this is consistent with the results from previous studies [13, 14] and with the attenuation nature of the PCV1-2 chimera virus [13].

In conclusion, the results from this study indicated that there was no evidence of reversion of the PCV1-2 chimera virus to its parental wild-type PCV1 or PCV2 virus after 11 serial passages in PK-15 cells. The PCV1-2 chimera vaccine virus is also genetically stable in pigs during the 3 *in vivo* serial passages. Therefore, the PCV1-2 chimera vaccine virus is suitable for further development into a potential live-attenuated vaccine against PCV2 infection.

2.5 Acknowledgements

2.6 References

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2.7 Tables

Table 1. Viremia of PCV1-2 chimeric vaccine virus identified by RT-PCR in serum samples during 3 serial passages in pigs

	Passage	Pig	DPI				
			0	7	14	21	28
Control pigs	1	177	-	-	-	-	-
		178	-	-	-	-	-
		179	-	-	-	-	-
	2	177	-	-	-	N/A	N/A
		178	-	-	-	N/A	N/A
		179	-	-	-	N/A	N/A
	3	82	-	-	-	-	-
		83	-	-	-	-	-
PCV1-2 Plasmid DNA group	1	180	-	-	-	N/A	N/A
		182	-	-	+	+	N/A
		181	-	-	-	+	-
	2	53	-	-	-	N/A	N/A
		51	-	-	-	-	N/A
		52	-	-	+	+	+
	3	77	-	-	-	N/A	N/A
		76	-	-	+	+	N/A
		79	-	-	+	+	+
PCV1-2 live virus group	1	185	-	+	+	N/A	N/A
		183	-	-	-	-	N/A
		184	-	+	-	+	+
	2	54	-	-	+	N/A	N/A
		56	-	-	+	+	N/A
		55	-	-	+	+	+
	3	80	-	-	+	N/A	N/A
		81	-	-	-	+	N/A
		78	-	-	+	+	+

DPI: Days post-inoculation
N/A: pig was necropsied on the previous DPI

Table 2. Occurrence of V79F marker back-mutation during serial passages in pigs inoculated with the chimeric PCV1-2 live virus as well as with the plasmid DNA of chimeric PCV1-2 infectious DNA clone

Inoculum	Passage	DPI	Occurrence of back-mutation in different tissues				
			Lung	MLN	SILN	IC	Tonsil
PCV1-2	1	14	-	-	-	-	-
Live Virus		21	-	-	-	‡	-
		28	-	V79F	V79F	V79F	V79F
	2	14	V79F	V79F	V79F	V79F	V79F
		21	N/S	N/S	N/S	N/S	N/S
		28	V79F	V79F	V79F	V79F	V79F
	3	14	N/S	N/S	N/S	N/S	N/S
		21	N/S	N/S	N/S	N/S	N/S
		28	V79F	V79F	V79F	V79F	‡
PCV1-2	1	14	-	-	-	-	-
Plasmid		21	-	-	-	‡	-
		28	-	-	-	-	‡
	2	14	‡	‡	‡	‡	‡
		21	‡	‡	‡	‡	‡
		28	V79F	V79F	V79F	V79F	V79F
	3	14	N/S	N/S	N/S	N/S	N/S
		21	N/S	N/S	N/S	N/S	N/S
		28	V79F	V79F	V79F	V79F	V79F

DPI: Days post-inoculation
N/S: Not Sequenced
‡:   Negative for PCV1-2 DNA by PCR
-:   No Mutation
MLN: Mesenteric lymph node
SILN: Superficial inguinal lymph node
IC: Intestinal Contents

2.8 Figures

Figure 1: Piglet grouping for three *in vivo* passages

Passage 1 (DPI)	Passage 2 (DPI)	Passage 3 (DPI)
	Recovered virus	Recovered virus	Recovered virus

PCV1-2 Plasmid DNA → Pig 1 (14), Pig 2 (21), **Pig 3 (28)**

Pig 7 (14), Pig 8 (21), **Pig 9 (28)**

Pig 10 (14), Pig 14 (21), Pig 15 (28)

PCV1-2 Live virus → Pig 4 (14), Pig 5 (21), **Pig 6 (28)**

Pig 10 (14), Pig 11 (21), **Pig 12 (28)**

Pig 16 (14), Pig 17 (21), Pig 18 (28)

Sterile PBS → Control 1, Control 2, Control 3

Control 5, Control 6
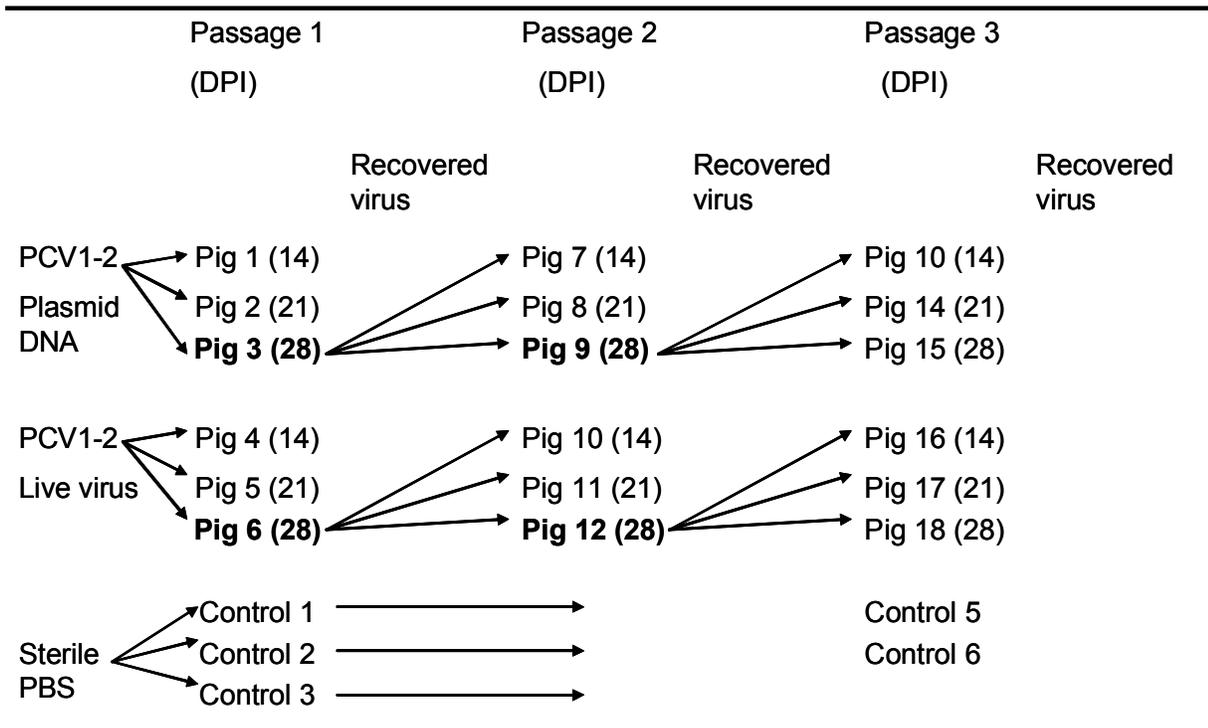
Tissue samples collected from highlighted pigs were used to inoculate new pigs in subsequent passage

General Conclusions

In conclusion, it was demonstrated that the PCV1-2 live vaccine is genetically stable in both PK-15 cell culture and in live pigs. It was shown that after 11 serial passages in PK-15 cells, not even a single nucleotide mutation was found in the vaccine virus genome. After 3 consecutive serial passages in live pigs, there was no evidence of mutation or reversion to the pathogenic form of the vaccine virus. It can be concluded that the vaccine virus DNA genome is genetically stable *in vitro* and *in vivo* and thus the PCV1-2 live vaccine, when it becomes available, should not pose major safety concerns in vaccinated swine herds.