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Horizontal transmission of porcine circovirus type 2 (PCV2)

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Horizontal transmission of porcine circovirus type 2 (PCV2)

by

Abby Rae Patterson

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
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    Ames, Iowa
    2010

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Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular, single stranded DNA virus of economic importance in the swine industry worldwide. The focus of this dissertation was to investigate different aspects of horizontal transmission including the use of serology to accurately detect infection, the infectivity and amount of PCV2 present in various secretions and excretions following experimental or natural PCV2 infection, the use of disinfectants in the prevention of PCV2 transmission on livestock trailers, the potential for spread of infectious PCV2 in spray-dried plasma, and evaluation of disinfection protocol of a swine facility following a natural PCV2 outbreak. Results of the first study indicated that evaluated ELISAs had area under the receiver operating curve values greater than 0.94, detected both anti-PCV2a and -2b antibodies with no differentiation, and did cross react with anti-PCV1 antibodies in infected animals. The second study determined that PCV2 exposure (natural or experimental) results in a chronic infection and PCV2 is shed in similar amounts by nasal, oral and fecal routes. In addition, PCV2 DNA present in excretions, secretions or on mechanical vectors is infectious to naïve pigs and therefore important for PCV2 transmission. The third study determined that although PCV2 DNA was detected on trailer surfaces by PCR following three different disinfection protocols, PCV2 was not transmitted to naïve animals exposed to the contaminated trailers. The fourth study provided direct evidence that an experimental spray-drying process was not effective in inactivating PCV2 and it is therefore possible that spray-dried porcine plasma from pigs could represent a biosecurity risk for the industry. The final study showed that the combination of a multistep
disinfection protocol with an improved, strict biosecurity plan can result in establishment of a PCV2 naïve herd and this status can be maintained for up to 10 months (300 days).
CHAPTER 1: General Introduction

DISSERTATION ORGANIZATION

This dissertation is prepared in an alternate manuscript format. The dissertation is composed of eight chapters and includes a general introduction, a literature review, five separate scientific manuscripts, and a general conclusion. References cited in the general introduction and the general conclusion chapters are listed at the end of the dissertation. The Ph.D. candidate, Abby Rae Patterson is the primary author of the manuscripts and is the principal investigator for the experimental work described. The included co-author’s on each paper provided input in the study design, facilitated animal work, or provided critical review of the manuscript prior to submission.

The first chapter of this work includes a general introduction to porcine circovirus type 2 (PCV2) followed by a section entitled “Statement of the Problem” which briefly summarizes the objectives and reasons for performing each study. The second chapter is a manuscript which provides a review of previously published literature on the epidemiology and horizontal transmission of PCV2 and has been submitted to Animal Health and Research Reviews. The third chapter is a manuscript which describes the diagnostic accuracy of three commonly used ELISAs for PCV2 and the ability of specific assays to differentiate between anti–PCV2a, anti–PCV2b, and anti–PCV1 antibodies. This manuscript was published in the Journal of Veterinary Diagnostic Investigation. The fourth chapter is a manuscript which describes the amount and infectivity of PCV2 shed in nasal, oral and fecal secretions following natural or experimental PCV2 infection. The manuscript has been submitted to Veterinary Microbiology. The fifth chapter is a manuscript which describes the ability of
PCV2 to withstand disinfection and be horizontally transmitted to naïve animals placed on livestock transportation trailers. The manuscript has been submitted to the *Journal of Swine Health and Production*. The sixth chapter is a manuscript which describes whether experimentally generated spray-dried plasma derived from a pig suffering from clinical porcine circovirus associated disease (PCVAD) was infectious in a swine bioassay using PCV2 naïve animals. This manuscript has been submitted to the *Journal of Animal Science*. The seventh chapter is a manuscript which documents a natural outbreak of PCV2 in a naïve population including a description of the procedures used to disinfect the premise and an attempt to derive PCV2 negative animals for use as replacement stock in the facility. This manuscript has been submitted to *Journal of Swine Health and Production*. The last chapter provides general conclusions.

**INTRODUCTION**

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular, single-stranded DNA virus that was associated with pigs exhibiting multisystemic wasting disease in the late 1990’s (Ellis et al., 1998). Since the initial description, PCV2-associated disease (PCVAD) has been reported in most pig producing countries worldwide and the virus is now considered ubiquitous (Ramamoorthy and Meng, 2009). PCVAD is now manifested in a variety of ways including multisystemic disease with wasting, reproductive failure, enteritis, respiratory disease, porcine dermatitis and nephropathy syndrome or a combination of these (Opriessnig et al., 2007). In cases of PCVAD, a morbidity rate of 4-30% with a mortality rate of 70-80% in affected animals has been reported (Segalés et al., 2005a). Commercial PCV2 vaccines are
now available and have generally been very effective in reducing morbidity and mortality in the field (Desrosiers et al., 2009) and under experimental conditions (Fenaux et al., 2004). To date, the question of how a highly prevalent virus such as porcine circovirus was and continues to cause epidemics of disease in populations of swine worldwide remains unanswered. The role of multiple co-infections, immune stimulation, immune suppression, management practices and many other factors in the development of clinical disease have been previously investigated (Gillespie et al., 2009; Madec et al., 2008; Opriessnig et al., 2007; Ramamoorthy and Meng, 2009). However, very little information exists on the role of horizontal transmission in the spread of PCV2 between pigs, farms, and countries.

**STATEMENT OF THE PROBLEM**

In order to accurately assess horizontal transmission of any pathogen, an assay with high diagnostic specificity and sensitivity is essential to detect when and if the pathogen is circulating in a herd. One useful, common and cost-effective method is to conduct a cross-sectional serological survey to determine the prevalence and levels of antibodies to the pathogen over time using one or more serological tests. For PCV2, most currently available ELISA tests use the open-reading frame (ORF) 2 region as an antigen since it is specific and immunogenic for PCV2 (Truong et al., 2001). Although most assays are based on PCV2 ORF2, the methods of antigen production can differ among assays (Blanchard et al., 2003; Liu et al., 2004; Walker et al., 2000; Wu et al., 2008). In addition, differences in the length of the PCV2 capsid protein used in the assay, antibody specificity, selection of cut-off value, or variability between technicians also increases variability between assays and between
diagnostic laboratories performing the same assay. While reports indicate that ELISAs based on the ORF2 region of PCV2 are useful, a direct comparison of the diagnostic accuracy of currently available ELISAs has not yet been performed to the authors’ knowledge. To address the gaps in knowledge on current PCV2 ELISAs, the following objectives were defined in Chapter 3: (1) to determine the diagnostic accuracy of 3 commonly used ELISAs, (2) to determine whether each assay can detect and/or differentiate between anti-PCV2a and anti-PCV2b antibodies, and (3) to determine whether PCV2–based ELISAs cross-react with anti–PCV1 antibodies.

Effective transmission depends on a number of factors falling under three main categories, characteristics of the host, characteristics of the pathogens and effective contact (Thrusfield, 2005). Specifically, the host must be susceptible; the pathogen must be virulent, infective and stable in the environment; and the interaction between the pathogen and host must be favorable for infection (correct infectious dose, adequate contact time, etc.) (Thrusfield, 2005). Previous studies which investigated both natural and experimental infection have reported detection of PCV2 DNA in various secretions and excretions including nasal and oral secretions and urine and feces (Bolin et al., 2001; Caprioli et al., 2006; Carasova et al., 2007; Grau-Roma et al., 2009; Segalés et al., 2005b; Shibata et al., 2003). However, the infectivity of the PCV2 in these samples has not been evaluated. The majority of work to date on transmission of PCV2 has focused on co-mingling of PCV2-infected and naïve animals and the infectivity of PCV2 in various secretions has not been assessed (Albina et al., 2001; Andraud et al., 2008; Bolin et al., 2001). In addition, the infection dynamics in naturally PCV2 infected segregated weaned PCV2 pigs or naïve pigs experimentally infected with PCV2 under controlled conditions has not been reported.
Therefore, the objectives of Chapter 4 were to determine the amount of PCV2 shed in nasal and oral secretions and feces following natural (Trial 1) or experimental (Trial 2) PCV2 infection and to demonstrate the infectivity of the PCV2 DNA detected in excretions, secretions or on the surface of a mechanical vector (needle) (Trial 3).

Knowledge of the modes of transmission of PCV2 is vital in understanding the ecology, epidemiology and control of PCVAD. While both vertical (Madson et al., 2009) and horizontal (Andraud et al., 2008) transmission by direct contact have been documented for PCV2, the focus of this thesis was on horizontal transmission by indirect contact. In general, horizontal transmission can occur by one of two routes. The first being direct contact with an infected host or the infected hosts secretions and the second, through indirect contact of a virus with a living vector, contaminated inanimate object or aerosol droplets (Thrusfield, 2005). As previous work has focused on direct contact (Dupont et al., 2009), Chapters 5 and 6 were focused on indirect transmission of PCV2. Key to both chapters is the hardiness of PCV2. Based on previous in vitro assays, PCV2 is quite stable following exposure to a wide range of temperatures (O’Dea et al., 2008) (Welch et al., 2006) and various pH conditions (Kim et al., 2009).

In Chapter 5, the ability of PCV2 to withstand disinfection and be horizontally transmitted to naïve animals placed on livestock transportation trailers was evaluated. Previous work suggests that under in vitro conditions, mean PCV2 titers were reduced but not consistently eliminated following exposure to various disinfectants (Kim et al., 2009; Royer et al., 2001). While the above information exists on the effectiveness of disinfectants under in vitro conditions, no information is available on the effectiveness of disinfectants under field conditions. A number of factors that need to be considered under field conditions...
that cannot be accurately recreated under in vitro conditions include the following: property of the surface that will be cleaned, water quality, and the presence of organic material contamination (Amass, 2004). Therefore, the hypothesis underlying the work performed in Chapter 5 was that the efficacy of various disinfectants against PCV2 is lower than described for in vitro work such that transmission of PCV2 through contaminated fomites is possible. To test this hypothesis, the specific objective of the study described in Chapter 5 was to evaluate the ability of four disinfection protocols with known in vitro effectiveness against PCV2 to prevent transmission of PCV2 under simulated field conditions.

Continuing in the theme of investigation of horizontal transmission of PCV2, another indirect method, spread of PCV2 in a contaminated feed product, was the focus of Chapter 6. The value of incorporating spray-dried plasma (SDP) protein into the diet of weanling pigs to improve feed intake and growth performance, especially following disease challenge has been well documented (Coffey and Crowell, 2001; Torrallardona, 2010). However, bacteria (DeRouchey et al., 2004), antibodies (Borg et al., 2002) and even PCV2 DNA (Pujols et al., 2008) can be detected in the spray-dried products. In the only study which has evaluated commercial SDP in regards to transmission of PCV2, PCV2 present in SDP was not found to be infectious. Specifically, commercially manufactured SDP protein containing $2.47 \times 10^5$ PCV2 genomic copies per ml fed to weaning pigs, which had maternally-derived PCV2 antibodies, did not result in seroconversion or viremia during the 45 day observation period (Pujols et al., 2008). As the animals used in the study were not serologically naïve to PCV2 and maternal antibodies can limit viremia and seroconversion in a titer dependent manner (McKeown et al., 2005), the hypothesis underlying Chapter 6 was that in immunologically PCV2-naïve animals, if infectious PCV2 is present in SDP this would be evidenced by
seroconversion and viremia. The specific objective of the study was to determine whether an experimentally generated SDP derived from a pig experimentally-infected with PCV2b was infectious in a swine bioassay using PCV2 naïve animals.

While animal work was ongoing for the data presented in the previous chapters, the swine herd supplying PCV2 negative animals for these studies underwent a natural PCV2 outbreak. As this is a fairly rare event due to high seroprevalence of PCV2 in multiplication herds worldwide, the situation presented a unique opportunity to implement knowledge gained in the previous studies on horizontal transmission on a large scale basis. The specific objectives of Chapter 7 were; therefore, to document a natural outbreak of PCV2 in a naïve population, to describe the procedure used to disinfect the premise, and to document the attempt to derive PCV2 negative animals for use as replacement stock in the facility.
CHAPTER 2. Literature review: Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2)

A paper submitted to Animal Health and Research Reviews

Abby Patterson, Tanja Opriessnig

ABSTRACT

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular, single stranded DNA virus of economic importance in the swine industry worldwide. Based on the sequence analyses of PCV2 strains, isolates can be divided into five subtypes (PCV2a-e). It is well documented that PCV2 is a ubiquitous virus based on serological and viremia data from countries worldwide. In addition, PCV2 DNA was discovered in archived samples prior to the documented onset of clinical disease. Recently a worldwide shift in PCV2 subtype from PCV2a to PCV2b has occurred. However, limited data exists on the horizontal transmission of PCV2. Previous studies have documented that transmission of PCV2 occurs by direct or nose-to-nose contact with infected animals and limited evidence to date also suggests that transmission through indirect contact is possible.

The objective of this review was to summarize data on the epidemiology and horizontal transmission of PCV2.
INTRODUCTION

Porcine circovirus (PCV), a small, non-enveloped, circular, single stranded DNA virus, was first discovered as a picornavirus-like contaminate of PK-15 cells in 1974 (Tischer et al., 1974). Although further studies on the virus indicated that it was non-pathogenic (Tischer et al., 1986), the discovery of the virus later designated PCV type 1 (PCV1) initiated worldwide investigations into the prevalence of the PCV in the swine population and led to the eventual discovery of PCV type 2 (PCV2). PCV2 is shares between 68-76% nucleotide sequence identity with PCV1 (Hamel et al., 1998; Morozov et al., 1998). Unlike PCV1, PCV2 was initially associated with clinical disease in the 1990’s (Allan et al., 1998; Harding et al., 1998) and today is of economic importance in the swine industry worldwide. Based on the sequence analyses of PCV2 isolates, they can be divided into five subtypes (PCV2a-e). PCV2a and PCV2b have been documented worldwide (Segalés et al., 2008), PCV2c has been documented in archived samples from Denmark (Dupont et al., 2008), and PCV2d and PCV2e have only been identified in China to date (Wang et al., 2009).

Clinical signs of PCV2 infection manifest in a variety of ways including multisystemic disease with wasting, reproductive failure, enteritis, respiratory disease, porcine dermatitis and nephropathy syndrome (PDNS) or a combination of these (Opriessnig et al., 2007) summarized as porcine circovirus associated disease (PCVAD). In cases of PCVAD, a morbidity rate of 4-30% with a mortality rate of 70-80% in affected animals has been reported (Segalés et al., 2005a). It is clear is that PCV2 is ubiquitous and was present many years prior to the onset of clinical PCVAD. To date, the question of how a highly prevalent virus such as PCV2 was able to cause epidemics of disease in populations of swine
worldwide remains unanswered. The presence of PCV2 prior to recognition of clinical PCVAD outbreaks in multiple countries indicates that the presence of co-factors contribute to the onset of clinical disease manifestation. Multiple factors including co-infections, immune stimulation, immune suppression, management practices and many others have recently been reviewed (Gillespie et al., 2009; Madec et al., 2008; Opriessnig et al., 2007; Ramamoorthy and Meng, 2009). To the author’s knowledge, an in depth review of the epidemiology and horizontal transmission of PCV2 has not been conducted to date.

EPIDEMIOLOGY

PCV2 is prevalent world-wide

Following the initial detection of PCV in 1974 (Tischer et al., 1974), the seroprevalence of this virus was determined in Germany (Tischer et al., 1986), Canada (Dulac and Afshar, 1989), England (Edwards and Sands, 1994) and in the United States (Hines and Lukert, 1995). Because these studies were published in the early 1990’s, the exact PCV type represented in the reports is unknown. Specifically, persistently infected porcine kidney cells (PK-15) were used for the immunofluorescent assay (IFA) making the differentiation of PCV1 and PCV2 difficult. Based on these initial studies it was determined that PCV was widespread in swine populations in the investigated countries (Table 1). By the late 1990’s however, diagnostic tests which differentiated non-pathogenic PCV1 from pathogenic PCV2 became available allowing further characterization of PCV isolates. PCV2 has since been identified in countries worldwide (Table 2).

PCV2 is identified in archived tissue samples prior to onset of clinical disease
Retrospective studies have indicated that PCV2 had been present years prior to the onset of clinical cases of PCVAD (Fig. 1). However, based on the limited sequencing information from these studies, little to no changes were detected in the viral genome between isolates recovered prior to and following clinical outbreaks of PCVAD (Grierson et al., 2004; Jacobsen et al., 2009). Interestingly, there has been a shift in the predominant PCV2 subtype to PCV2b which may have contributed to the increased severity and frequency of PCVAD.

**A global shift in the predominant PCV2 subtype**

Several studies have evaluated the temporal differences in identification of PCV2a and PCV2b and concluded that a global shift in the predominant subtype has occurred. In a study published in 2007, 218 PCV2 genome sequences were analyzed and the authors’ concluded that a major shift from PCV2a to PCV2b occurred on a global scale in or prior to 2003 (Dupont et al., 2008). Interestingly, based on the analysis in the study which included sequences from 1997 to 2006, the shift in subtypes did not affect Korea, Japan or Australia (Dupont et al., 2008). Since that time, PCV2b has been demonstrated as the predominant subtype in Korea based on analysis of isolates from 2005-2007 (Kim et al., 2009b) and in Japan where both PCV2a and PCV2b were detected in isolates collected between 2006 and 2007 (Takahagi et al., 2008). Additionally in a study by Olvera et al., 148 PCV2 isolates were analyzed. Of these, 63.5% (94/148) were PCV2b sequences of which 92.5% (87/94) were published after 2003. Numerically fewer (62.2%; 33/53PCV2a sequences were published after 2003 (Olvera et al., 2007). However, the authors’ cautioned that the time of sequence publication in relationship to when the virus was first detected can be variable.
In the following section, summaries of selected reports which document the predominance of the PCV2b subtype are provided for each continent.

**Asia**

In China, 49 isolates from various geographic regions were sequenced (Wang et al., 2009). Results revealed that only PCV2a isolates were recovered in 2001 (n=3). In isolates recovered between 2002 and 2006 (n=34), a mixture of PCV2a, PCV2b, PCV2d and PCV2e was detected with the majority of samples (n=18) clustering with PCV2b. Of the isolates recovered in 2007 and 2008 (n=12), all clustered with PCV2b (Wang et al., 2009). Similarly, in a 2010 publication evaluating a total of 136 isolates from Eastern China collected between 2001 and 2009, 127 of the isolates clustered with PCV2b (Li et al., 2010), while only nine clustered with PCV2a. Of these nine, eight were collected between 2001 and 2004. The remaining 48 isolates collected during this period were identified as PCV2b (Li et al., 2010).

In Korea, PCV2 isolates detected by PCR in tissue samples collected from PCVAD affected herds (n=29) and non-PCVAD affected herds (n=9) in 2007 were sequenced and categorized as PCV2a and PCV2b. All of the 29 sequences from PCVAD affected herds and four of the sequences from non-PCVAD affected herds clustered into the PCV2b group (Chae and Choi, 2009). Similarly, in a study by An et al. (2007) 36 Korean PCV2 sequences from pigs with PDNS or PCVAD collected from 1999 to 2006 were compared to other PCV2 sequences available in GenBank. The phylogenetic analysis indicated that PCV2b was detected in 66.7% (24/36) of the samples. Although the specific year when these 24 isolates were
collected is not provided, the authors’ suggest that more severe PCVAD cases in Korea in 2004 were related to the increased incidence of PCV2b (An et al., 2007).

In Japan, 150 serum samples were collected from 2- to 4-month old pigs on 10 farms (15 samples/farm). PCV2 DNA positive samples were analyzed by restriction fragment length polymorphism (RFLP) analysis. In two farms, RFLP type 311 (which corresponds to PCV2a) was identified in 2006. Six months later in the two farms, RFLP type 111 (which corresponds to PCV2b) was identified indicating a shift in the PCV2 subtype over a short temporal span (Takahagi et al., 2008).

*Europe*

In Ireland, PCV2a was recovered from five of six farms classified as PCVAD-affected based on samples collected from 1997 to 2003 (Allan et al., 2007). However, PCV2b was isolated from the remaining farm which had increased mortality (peak mortality of 35%) in comparison to the other five farms. In all samples after 2003 from both PCVAD-affected and non-PCVAD-affected farms, only PCV2b was isolated (Allan et al., 2007). As severe PCVAD outbreaks occurred between 2003 and 2006 in Ireland, this is suggestive of a temporal link between the identification of PCV2b and severe outbreaks (Allan et al., 2007).

In Brittany, de Boisséson et al. (2004) sequenced and compared isolates from PCVAD (n=25) and non-PCVAD (n=31) affected farms collected from 2000 to 2002. Results indicated that two main clusters were present with the majority of the PCV2 sequences clustering into the PCV2b group (de Boisséson et al., 2004). While the author’s concluded
that a new subtype of PCV2 was not responsible for the increased outbreaks of PCVAD in Brittany as PCV2b was detected in both PCVAD and non-PCVAD affected herds (de Boisséson et al., 2004), this is consistent with PCV2b being the predominant strain during this time.

In Denmark, Dupont et al. sequenced and compared a total of 45 isolates from PCVAD affected and non-PCVAD affected herds collected between 2003 and 2004. Similar to the study by de Boisséson et al., a distinctive clustering of isolates by disease status was not noted; however, all isolates clustered within the PCV2b group (Dupont et al., 2008).

**North America**

In North America, the shift to increased prevalence of PCV2b was documented by several recent studies. The first identified the presence of PCV2b in Canada in samples from 13 PCVAD cases collected in 2005 in the province of Quebec (Gagnon et al., 2007). Further investigation of 83 sequences collected from both PCVAD affected and non-PCVAD affected animals from four provinces in Canada between 2005 and 2006 revealed that 79.5% (66/83) of sequences clustered with PCV2b (Gagnon et al., 2007). In a separate study, 121 cases from animals with and without clinical signs of PCVAD submitted to the Institut National de Santé Animale between October 2006 and January 2007 were analyzed by a PCV2a/PCV2b differential PCR (Gagnon et al., 2008). Of the 121 cases, PCV2b was detected in 92.6% (112/121), PCV2a in 4.1% (5/121) and both subtypes were detected in 3.3% (4/121) of cases (Gagnon et al., 2008). Another publication identified 12 PCV2b isolates in four finishing facilities in Kansas experiencing severe PCVAD (Horlen et al.,
PCV2b was also identified in tissue homogenates from pigs manifesting severe PCVAD in Kansas, North Carolina and Iowa in 2005 (Cheung et al., 2007). Previous to these reports, PCV2a had been the only strain detected in North America (Fenaux et al., 2000; Larochelle et al., 2002; Olvera et al., 2007). However, these reports included sequence information from limited numbers of North American isolates.

**Oceania**

The paucity of sequencing information available on Australian and New Zealand isolates limits drawing conclusions on whether a shift in predominant subtype occurred in this region or what the predominant subtype currently is. To the author’s knowledge, there are only two studies evaluating isolates from this region. In the first, Muhling et al. evaluated seven PCV2 isolates collected between 1999 and 2002. Six of the isolates clustered with previous isolates from Japan and Canada and had an overall 94-99% nucleotide identity with previous reported strains from other countries (Muhling et al., 2006). Specific designations of the isolates into PCV2a and PCV2b were not made. The second study evaluating isolates from New Zealand found that isolates from the 2006 PCVAD outbreak share 94-100% nucleotide identity with other reported PCV2 isolates (Neumann et al., 2007).

**South America**

In tissues from pigs with respiratory signs collected between 2000 and 2004, eight PCV2 sequences were identified (Castro et al., 2008). In a later study by Chiarelli-Neto et al. (2009) 11 Brazilian isolates from PCVAD-affected animals collected in 2005 were sequenced. The majority of Brazilian isolates clustered with the PCV2b group and the
author’s concluded that Brazilian strains emerged from a common ancestor identical or descendent from strains which were common in Europe and Asia (Chiarelli-Neto et al., 2009). While this provides information that PCV2b is currently likely the predominant subtype, it does not determine when or if a shift occurred. The information for other South American countries is limited (Cano et al., 2005).

While broad and firm conclusions from the above referenced studies cannot be made because of the low numbers of isolates evaluated (especially those collected prior to 2005), the available data suggests that PCV2b subtypes currently predominate. Additional sequences, especially from isolates collected prior to 2005, are necessary to determine a more precise estimate of the time when the shift in subtypes occurred. Interestingly, while the studies in which both PCVAD-affected and non-affected animals were evaluated support an increased identification of PCV2b, they do not necessarily support the association of clinical disease with PCV2b as this subtype was found in both clinically affected and non-affected animals.

**HORIZONTAL TRANSMISSION**

Effective transmission depends on a number of factors falling under three main categories, (1) characteristics of the host, (2) characteristics of the pathogens and (3) the type of exposure (Thrusfield, 2005). Specifically, the host must be susceptible; the pathogen must be virulent, infective and stable in the environment; and the interaction between the pathogen and host must be favorable for infection (correct infectious dose, adequate contact time, etc.) (Thrusfield, 2005). In general, horizontal transmission can occur by one of two routes, direct contact with an infected host or the infected host’s secretions or through indirect contact of a
virus with a living vector, contaminated inanimate object or aerosol droplets (Thrusfield, 2005). The following focuses on routes of PCV2 shedding, environmental stability, and evaluation of the transmission of PCV2 through direct and indirect contact.

**PCV2 is shed in large quantities through multiple routes**

Following experimental infection of naïve animals with PCV2, PCV2 DNA has been detected in multiple sample types. Caprioli et al. (2006) oronasally inoculated 4 week old colostrum-deprived (CD) specific pathogen free (SPF) pigs with a PCV2 isolate recovered from a clinically affected Canadian pig. Blood, fecal swabs and tonsillar swabs were collected at 1, 3, 6, 9, 12, 15, and 21 days post inoculation (dpi). Results indicated that 10/12 and 11/12 pigs had PCV2 DNA-positive tonsillar and fecal swabs, respectively, at 1 dpi. Interestingly, at dpi 6, only 1/12 and 0/12 animals were PCV2 DNA positive on tonsillar and fecal swabs, respectively. However, by the last day of the trial (dpi 21), 11/12 and 11/11 animals were PCV2-positive by tonsillar and fecal swabs, respectively. Additionally, PCV2 DNA was not detected in serum, plasma or whole blood until 9 dpi. Based on these findings, the authors’ concluded that PCR had detected inoculum in the nasal and fecal swabs prior to dpi 6. In a separate study, Bolin et al. (2001), intranasally and subcutaneously inoculated mixed breed, cesarean-derived, colostrum-deprived (CDCD) pigs at 20 or 25 days of age with a PCV2 isolate obtained from a clinically affected Canadian pig to determine tissue distribution of PCV2 DNA and duration of viral persistence (Bolin et al., 2001). Virus was recovered by isolation from nasal, rectal or tonsil swabs between 12 and 19 dpi (Bolin et al., 2001). Analysis of shedding in CDCD pigs was also evaluated by Shibata et al. in 2003. In this study, pigs were intranasally inoculated with PCV2 at 2 weeks of age and oropharyngeal
and nasal swabs and feces were collected among other samples at the time of necropsy (necropsy occurred on dpi 7, 14, 21, 35, 49, or 70). Results indicated early and consistent shedding in the majority of animals for an extended period of time. All oropharyngeal and nasal swabs and fecal samples contained PCV2 DNA on dpi 1 (excluding 1 nasal swab). Between dpi 1 and dpi 70 all fecal samples were PCV2 PCR positive and 83 out of 86 nasal and oropharyngeal swabs were PCV2 DNA positive (Shibata et al., 2003). In a similar study in which CDCD pig were inoculated with PCV2, PCV2 DNA was detected in nasal and rectal swabs in a high percentage of sampled animals (67-100% excluding one nasal sampling at dpi 35 in which only 1 of 3 animals had detectable PCV2 DNA) between 7 and 49 dpi (Harms et al., 2001). Several other studies detected PCV2 DNA or viable virus (by virus isolation) from nasal swabs, feces, ocular swabs or a combination of these samples at various time points in low numbers of animals following experimental infection (Krakowka et al., 2000; Magar et al., 2000a; Resendes et al., 2002).

Limited data on the amount of PCV2 in nasal fluid and saliva following experimental infection is available. Chung et al. (2005) intranasally challenged 14 cross-bred commercial pigs which were serologically naïve to porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 at 10 weeks of age with a tissue homogenate containing PRRSV and PCV2 (n=12) or animals were left as negative controls (n=2). As part of the study, six of the animals challenged with PCV2 and PRRSV were injected intramuscularly with inactivated Salmonella choleraesuis and complete Freund’s adjuvant prior to infection. In experimentally infected pigs PCV2 concentrations at 14 dpi were similar to those found in naturally infected animals. Samples were collected from nine dyspneic pigs naturally infected
with PRRSV and PCV2 (based on PCR detection in serum) and nasal fluid and saliva of these pigs contained between 3.7±0.2 to 4.4±0.2 log_{10} PCV2 genomic copies/ml (Chung et al., 2005).

While the above studies included relatively low numbers of pigs with varying immune status (CDCD, CD or commercial SPF pigs) and pigs were inoculated with different PCV2 isolates, PCV2 DNA was consistently detected in the secretions and excretions evaluated. In addition, PCV2 DNA was detected early in the infection process (dpi 1) and generally persisted in a high number of the animals through the end of the evaluation period indicating persistent infection. To the author’s knowledge, there is only one study to date investigating the actual amount of PCV2 DNA in various secretions following experimental infection (Chung et al., 2005) and more work in this area is needed to determine whether shedding occurs equally by various routes.

Additional studies have described PCV2 shedding following natural infection of pigs and PCV2 was detected and quantified in multiple sample types. The PCV2 shedding patterns in three naturally PCV2 infected farms was investigated between 1999 and 2000 (Shibata et al., 2003). Nasal swabs and fecal samples were collected from 313 pigs in five different age groups. Results suggested that infection occurred post-weaning as an increased frequency of PCV2 detection occurred in both sample types after the nursery phase (n=64), peaked in 3-4 month old animals (n=81) and declined in the sampled population of adult animals (n=47). Overall, PCV2 was detected in 20.4% (64/313) of fecal samples and 19.2% (60/313) of nasal samples (Shibata et al., 2003). In 2009, a longitudinal case-control study of two PCVAD
farms located in Spain and Denmark was designed to describe the dynamics of a natural PCV2 infection (Grau-Roma et al., 2009). In contrast to the previously described cross-sectional study, this study provided data on a specific cohort of animals over time. Serum, nasal swabs and fecal swabs were collected from selected animals at four time points from 1 to 9 (Denmark) or 11 (Spain) weeks of age and at the time of necropsy (between 9 to 21 weeks of age). Of the 108 Spanish animals evaluated, 78 animals were defined as “case” and 30 as “controls” based on clinical evaluation. A similar clinical evaluation resulted in 91 case and 27 control animals from farms in Denmark. On Spanish farms, PCV2 was detected by PCR sporadically in all samples between week 1 (first sampling point) and week 11. At the time of necropsy, PCV2 was detected in 87.0% of nasal and 50.5% of fecal swabs. A significantly higher percentage of positive pigs were detected in animals classified as “case” animals. The amount of PCV2 DNA in nasal and fecal samples ranged from approximately 4-6 log_{10} genomic copies PCV2 per swab throughout the study period with significantly higher amounts detected in “case” animals. In Danish farms at one week of age, 69.3% of nasal swabs and 22.8% of rectal swabs were PCV2 DNA positive. By the time of necropsy, the percentage of PCV2 positive PCR samples had risen to 100% in nasal samples and to 98.5% of rectal swabs. Throughout the study period, the amount of PCV2 DNA ranged from approximately 4 to 8 log_{10} genomic copies PCV2 per swab in fecal samples and 4 to 10 log_{10} genomic copies PCV2 per swab in nasal samples. By the time of necropsy, “case” animals had significantly larger amounts of virus in both nasal and fecal swabs in comparison to healthy controls (Grau-Roma et al., 2009). Quantitative data on the amount of PCV2 DNA in various samples collected from 146 pigs submitted to the Pathological Diagnostic Service at the Veterinary School of Barcelona between 2000 and 2001 was reported in 2005 (Segalés
Serum samples (n=57), nasal swabs (n=99), tonsillar swabs (n=108), tracheobronchial swabs (n=72), urinary swabs (n=91) and fecal swabs (n=42) were collected and were tested quantitatively for the presence of PCV2 DNA. The results were divided into three categories: PCVAD affected pigs, PCV2 subclinically infected pigs and non-PCVAD pigs, based on presence of histopathological lesions and amount of PCV2 antigen detected by in-situ-hybridization and into two categories based on age (≥1.5 or less than 1.5 months of age). PCV2 was found in 100% of all swabs and serum samples from animals classified as PCVAD affected pigs with significantly more PCV2 DNA present in comparison to PCV2 subclinically infected pigs and non-PCVAD pigs. Additionally, significantly more PCV2 DNA was present in older animals (≥1.5 months of age) except for tonsillar swabs. Quantitative results for PCVAD pigs ranged from approximately 5 to 8 log_{10} PCV2 genomic copies/ng for all sample types with the highest PCV2 load detected in trachea-bronchial swabs followed by serum, tonsillar, nasal, fecal and urinary swabs. In all sample types, the amount of PCV2 was higher in PCVAD affected pigs compared to non-PCVAD pigs. The authors’ concluded based on these results that the more severe the PCV2 lesions, the more PCV2 is present within serum and various swabs (Segalés et al., 2005b).

While larger numbers of animals were used in studies evaluating naturally PCV2 infected pigs, the impact of management, co-infections and other co-factors in PCV2 shedding are difficult to evaluate. However, the above studies suggest that PCV2 is present in high quantities in nasal and fecal samples for extended periods of time following natural PCV2 infection of a population. In addition, there is evidence that increased shedding is present in animals with clinical signs associated with PCV2 in comparison to healthy controls (Grau-
Roma et al., 2009). Similar to the limited data available on the amount of PCV2 present in secretions from experimentally infected animals, few studies have evaluated this aspect following natural infections.

In summary, the above information suggests that PCV2 DNA can be detected in fecal, nasal, oral, and tonsillar swabs as well as in urine and feces from both naturally and experimentally infected pigs. In addition, PCV2 DNA can be detected early in the infectious process and persists for extended periods of time. However, in the majority of the studies, the infectivity of the PCV2 DNA was not investigated.

**PCV2 is stable in the environment and resistant to disinfectants**

Relatively few studies have evaluated the stability of PCV2; however, available results indicate that the virus is very resistant under high temperatures and a wide range of pH conditions. In a study by Welch et al. (2006), PCV2 virus stock was subjected to one of two treatments. The first treatment was pasteurization at 60°C for 0, 2, 5, 10 and 24 hrs and the second treatment was dry heat treatment on freeze-dried virus (selected vials were partially rehydrated) at 80°C for 8, 24, 48 or 72 hrs. Following application of the treatments, an infectivity reduction factor was calculated. For pasteurization, a 1.33 log reduction (corresponding to 5% of the starting material) was calculated at 24 hrs. Following dry-heat treatment, a 0.75 log reduction (corresponding to 18% of the starting material) was calculated at 72 hrs. Due to the lack of efficacy of temperatures utilized, additional temperatures were evaluated. Results indicated pasteurization at a temperature of 65°C, 70°C and 75°C for 30 min lead to a 0.25, 1.59 and 1.92 log reduction, respectively. Interestingly, only a 1 log
reduction in infectivity was calculated following dry-heat treatment at 120°C for 30 min (Welch et al., 2006). Resistance of PCV2 to heat was also shown in a study by O’Dea et al. in which PCV2 virus stock in cell culture media was incubated in a water bath at temperatures of 56, 65, 70, 75, 80, and 85°C (O’Dea et al., 2008). The presence of PCV2 antigen following treatments was determined by immunohistochemistry (IHC) and the presence of replicating PCV2 was determined by RT-PCR for replicative intermediates. Results indicated that PCV2 antigen was detected at temperatures up to 70°C and PCV2 RNA was detected up to 75°C but not at 80°C. The author’s suggested that PCV2 was more labile when heated in liquid culture then when freeze-dried explaining the differences noted between this study and previous work by Welch et al (O’Dea et al., 2008; Welch et al., 2006). In 2009, the in vitro stability of PCV2 was again investigated by incubating PCV2 at various temperatures and ranges of pH. Results confirmed extreme environmental resistance as evidenced by an unchanged virus titer following incubation at 56°C for one hour, a reduction from $10^{5.5}$ to $10^{2.5}$ 50% tissue culture infective dose (TCID$_{50}$) per ml following incubation at 70°C for one hour, and a reduction from $10^{5.5}$ to $10^{2.7}$ TCID$_{50}$ per ml following incubation at a pH of 2 for 30 minutes (Kim et al., 2009a).

In addition to showing environmental stability, PCV2 has been shown to withstand several types of disinfectants. In a study by Kim et al., various disinfectants were evaluated for efficacy against PCV2 in an in vitro model. Specifically, incubation of a potassium peroxomonosulphate, a sodium hypochlorite product, or 3% sodium hydroxide with $1 \times 10^{4.5}$ to $1 \times 10^{5.5}$ TCID$_{50}$/ml for 10 min resulted in complete reduction of PCV2 (Kim et al., 2009a). When the incubation time was increased to 30 min with a quaternary ammonium
compound or 12 hrs with a quaternary ammonium/gludaraldehyde compound there was also
a complete reduction of PCV2 infectivity (Kim et al., 2009a). When PCV2 was incubated
with 0.05 or 0.1% formalin, PCV2 was detected; however, a specific mean TCID$_{50}$/ml was
not provided (Kim et al., 2009a). In an earlier study, an in vitro suspension assay was
performed to determine the virucidal efficacy of nine disinfectants against a French PCV2
isolate (Martin et al., 2008). Equal volumes of double strength disinfectant were mixed with
PCV2 virus stock for 30 min at 20°C. Results indicated a reduction of the mean PCV2 titer
of greater than 1.61 log$_{10}$ at the authorized concentration for the two disinfectants which
contained potassium monopersulfacte as the active ingredient and the oxidizing agent with a
combination of peracetic acid and hydrogen peroxide as active ingredients. Products
containing a combination of aldehydes and quaternary ammonium active ingredients resulted
in a reduction greater than 1.74 log$_{10}$ of the PCV2 titer. Products containing iodine and
phenolic compounds as active ingredients did not reduce the infectivity titer of PCV2. While
results indicate several of the products evaluated reduced the infectivity titer of PCV2, none
of the products resulted in a 3 log$_{10}$ TCID$_{50}$/ml reduction in infectivity titer (French
regulation for determination of virucidal efficacy) due to the low initial titer of the PCV2
used in the suspension (Martin et al., 2008). In a similar study by Royer et al. (2001), PCV2
virus stock was incubated at room temperature for 10 min with the recommended dilution of
various disinfections, passed through a detoxification column and assessed for infectivity by
cell culture. Results indicated several disinfects significantly reduce the mean infectivity tiers
of PCV2 following exposure including two phenol products (30.6-40.3% mean reduction
titer), two quaternary ammonium compounds (34.7-50.0%), two oxidizing agents (45.8-
73.6%) and an alkali agent (61.1%). However, products containing alcohol, iodine,
formaldehyde and chlorhexidine compounds did not result in a significant reduction in mean infectivity titer. While several products were efficacious, none of the products completely eliminated infectious PCV2 under optimal, laboratory conditions (i.e. no organic material present, optimal contact time, optimal physical surface) (Royer et al., 2001).

Taken together, these studies provide evidence that reduction of PCV2 titers under in vitro conditions is most likely to occur when an oxidizing, halogen, or sodium hydroxide containing product is used while products containing iodine, alcohol, phenol or formaldehyde are likely to be less effective. Complete inactivation of PCV2 under in vitro conditions is difficult (only accomplished in 1 of 3 studies) and results are difficult to interpret as neither a cytotoxicity control nor a virus control (virus plus media alone) were used in the study. Lastly, all published work with disinfectants has been done under in vitro conditions and it is unknown if these results would be repeatable under in vivo conditions.

**Documentation of horizontal transmission of PCV2 by direct contact with infected animals**

The majority of evidence to date on the transmission of PCV2 by direct or nose-to-nose contact comes from investigations where comingling of infected pigs with naïve animals was done following experimental infection. Bolin et al. comingled mixed-breed, CDCD pigs which were intranasally and subcutaneously inoculated at 20 or 25 days of age with a Canadian PCV2 isolate with naïve contact pigs. Contact pigs were comingled at 42 dpi and all three contact pigs seroconverted within 3 weeks of contact exposure (Bolin et al., 2001). In a similar study, six 10-13 week old pigs from a PCVAD affected conventional farm were
comingled with 10 week old specific pathogen free pigs (Albina et al., 2001). Following com-
ingling, 5 of 5 contact pigs developed clinical signs including pyrexia and diarrhea.

Additionally, separate groups of 5-9 week old SPF or conventional pigs were inoculated with a tissue homogenate derived from pigs showing clinical signs of PCVAD. PCV2-naïve contact animals were comingled with these animals immediately following experimental inoculation. Ninety percent of contact animals (10 of 11 pigs) developed pyrexia and growth retardation. These clinical signs were also seen in 82% (45/55) of the inoculated animals. Severe wasting was noted in 7.2% (4/45) of the inoculated animals and was not noted in contact animals. The onset of disease in contact animals was approximately 16 days following the onset of clinical disease in inoculated animals (Albina et al., 2001).

While the above studies documented that naïve animals can be infected with PCV2 following exposure to infected animals, low numbers of animals were used and information on specific transmission parameters including latency, basic transmission rate and mean disease generation time were not determined. The incubation period was found to be approximately 16 days in one study (Albina et al., 2001); however, the information was based on only four animals.

Several aspects of transmission have been evaluated by Andraud et al. (2009) using a time-
dependent transmission model. Using experimentally infected pigs comingled with naïve contact pigs in six successive transmission trials, the latency period for PCV2 was estimated to be 8 days, the basic reproduction ratio was estimated to be 5.9 and the mean disease generation time was estimated to be 18.4 days. Interestingly, in the study, the transmission
rate decreased between 15 and 55 dpi even though pigs remained viremic with viral loads between $10^3$-$10^6$ genomic copies/ml. This suggested a correlation between the presence of neutralizing antibodies and decreased transmission secondary to reduced viremia.

Alternatively, the infectious period did not correspond to the period of viremia (Andraud et al., 2009). In a second study by the same research group, further modeling was done to determine specific transmission parameters including the basic reproduction ratio ($R_0$), within pen transmission rate ($\beta_{\text{within}}$) and the transmission rate between pens ($\beta_{\text{between}}$) by comingling experimentally infected and naïve animals (Andraud et al., 2008). A maximum likelihood model including a constant six day latent period (based on previous in vitro infectivity titer data) and an assumption of the duration of infection were used. Under the assumption that pigs were “recovered” following seroconversion and decreased PCV2-viremia, the within pen $R_0$ was 8.9 (5.1-15.4) and $\beta_{\text{within}}$ was 0.28 (0.18-0.42). Using the same assumptions, the $R_0$ between-pen was 1.2 (0.5-2.9) and the $\beta_{\text{between}}$ was 0.04 (0.02-0.08). In the between pens or indirect contact group, the first viremic contact pig was detected at 17 days post comingling; by the first necropsy date (35 days), 16/16 pigs were viremic. In this group, seroconversion was first noted at 35 days; 3/16 pigs had seroconverted at the time of either the first or second necropsy (35 and 42 days, respectively). In the within pen or direct contact group, viremia was first detected at 21 days and all 16/16 pigs were viremic by 35 days post exposure. In this group, seroconversion was first detected at 35 days and 7/16 pigs were positive at either the first or second necropsy date. These results suggest that movement of PCV2 between pens is expected to be low, and hence the risk of movement of PCV2 between farms without direct contact to PCV2 infected pigs or fomites is low.

Combined with information on age susceptibility to PCV2, the authors’ suggested that
segregation of pigs from different sources until the risk of PCV2 infection decreases [more than 7 weeks; (Rose et al., 2009)] would likely result in decreased incidence of clinical disease associated with PCV2 (Andraud et al., 2008).

While these studies provide the only available information on transmission parameters for PCV2, they are based on low numbers of animals using a single replicate. For example the information for the direct contact group is based on a single replicate of four experimentally infected PCV2 animals which were comingled with four PCV2 naïve animals for a two day contact period. Assumptions in the model included homogenous infectious potential of experimentally infected animals and random mixing of the experimentally infected pigs and naïve contact animals. These assumptions may or may not be valid under field conditions. In addition, as noted by the authors, these results were generated from subclinically infected animals whereas results may differ in animals experiencing systemic clinical manifestations of PCVAD (Andraud et al., 2009).

In addition to providing evidence that PCV2 can be transmitted from pig to pig, the ability of PCVAD to be “spread” between groups of pigs has been evaluated. One such investigation comingled 108, 6-11 week old pigs from four herds defined as PCVAD-affected with 216, 4-5 week old pigs from two non-affected herds for 46-48 days. Pigs were divided into four compartments to evaluate direct contact, nose-to-nose contact between pens and no contact (separation by an aisle). In each compartment there were two pens containing comingled pigs from PCVAD-affected and non-affected herds (direct contact), one pen of non-affected pigs with nose-to-nose contact to the comingled pigs, one pen of pigs from PCVAD-affected pigs
in nose-to-nose contact with comingled pigs and one pen of non-affected animals with no contact (Dupont et al., 2009). Compartments had concrete floors with straw bedding and partitions between pens allowed nose-to-nose contact and movement of feces between pens (Kristensen et al., 2009). Disinfection was done with formaldehyde prior to initiation of the study with 2 week downtime without pigs (Kristensen et al., 2009). PCVAD was diagnosed by histopathology and immunofluorescence in 44 of the 324 pigs included in the study. Of the 44 PCVAD animals, 30 pigs were from PCVAD-affected herds and 14 were from non-affected herds. Transmission was confirmed by comparing PCV2 sequence data within individual animals (prior to the trial and following exposure) and among all animals. Sequencing results indicated that of the 14 animals from non-affected herds, eight were infected through direct contact, four from nose-to-nose contact, and two were infected without direct contact to infected animals. On-site control animals (housed in a separate compartment did not develop clinical disease; however, PCV2 genomic copies per ml serum increased from $10^4$ to $10^9$ during the study in two animals indicating exposure by an indirect means (Dupont et al., 2009). During the study, personnel changed gloves, clothes and boots between pens (Kristensen et al., 2009). Based on this work, the transmission of PCV2 with the subsequent development of clinical PCVAD can occur following comingling or nose-to-nose contact of animals from PCVAD-affected and non-PCVAD affected farms.

Overall, information on horizontal transmission of PCV2 comes from studies in which PCV2-naïve animals are exposed to experimentally or naturally PCV2-infected animals for a minimal contact period. Although in the majority of studies small numbers of animals were
used, it is clear that PCV2 can be transmitted to naïve animals by direct or close contact. However, more information is necessary to determine basic transmission parameters.

**Limited, inconclusive information is present on indirect transmission of PCV2 by the oral route, aerosol route and contact with other species**

*Transmission of PCV2 by the oral route*

Published information to date suggests that PCV2 may be infectious via the oral route as PCV2-naïve animals became infected following oral consumption of uncooked meat from an experimentally infected pig (Opriessnig et al., 2009b). In this study, skeletal muscle, bone marrow and lymphoid tissues collected from experimentally PCV2-inoculated pigs transmitted PCV2 by the oral route to naïve animals evidenced by seroconversion and viremia. Tissues were collected from animals at the time of peak infection and fed raw to naïve animals for three consecutive days. PCV2 genomic copies in the inoculum were $10^{7.8}$ and $10^{5.8}$ in lymphoid tissues and bone marrow, respectively. Interestingly, skeletal muscle, which is not a commonly demonstrated source of PCV2, contained $10^{4.0}$ PCV2 genomic copies/ml and was able to transmit PCV2 by the oral route to naïve animals. It was not determined whether the low levels of PCV2 were due to residual blood within the muscle or the presence of the virus within endothelial cells. Additionally, it was noted in the study that seroconversion (both anti-IgM and anti-IgG responses) was delayed in pigs inoculated with skeletal muscle and bone marrow suggesting that a lower infectious dose may result in delayed infection (Opriessnig et al., 2009b). Based on this study, it is known that PCV2 can be transmitted through oral consumption of uncooked meat; however, delayed seroconversion suggests that this route is not as efficient as direct contact with infected
animals. In addition, the significance of this route of infection in animals with passive or active immunity to PCV2 remains unknown.

Limited work has been done on indirect transmission of PCV2 through contaminated feed additives. In a study by Fenaux et al. (2004), a porcine-derived commercial pepsin product was found to contain PCV2 DNA (Fenaux et al., 2004). However, both in vitro analysis and a bioassay indicated the PCV2 detected by PCR was non-infectious. The bioassay portion of the study included 16, 5 week old SPF pigs of which six were intranasally and intramuscularly inoculated with the reconstituted commercial pepsin product. Evaluation of animals for clinical signs, seroconversion and viremia was done for 42 dpi. (Fenaux et al., 2004).

In another study, oral transmission of PCV2 via spray-dried plasma was evaluated. Numerous studies have documented the value of incorporating spray-dried plasma protein into the diet of weanling pigs to improve feed intake and growth performance, especially following disease challenge (Coffey and Crowell, 2001; Torrallardona, 2010). However, due to the inherent stability of PCV2, withstanding the spray-drying process is possible. To date, one study has evaluated the presence of PCV2 in spray-dried plasma. In this study, pigs with low levels of maternal PCV2 antibody were fed a diet containing 8% of a commercial spray-dried plasma product which contained $2.47 \times 10^5$ PCV2 DNA copies/ml for 45 days (Pujols et al., 2008). Results of weekly PCV2 PCR and ELISA analysis on serum samples indicated neither treated nor control pigs became viremic or seroconverted to PCV2 during the trial period. Based on the results, the authors suggested that spray-dried plasma containing PCV2
DNA fed to pigs with low levels of maternal antibodies represents a low risk of infection (Pujols et al., 2008). However, it is unknown whether the animals were susceptible to infection as a PCV2 challenge control was not included in the study. Previously, in animals with high levels of passively-acquired antibodies, PCV2 viremia and seroconversion were decreased following experimental challenge with PCV2; however, low levels of passively acquired antibody titers were not found to be generally protective (McKeown et al., 2005). As anti-PCV2 antibodies can mask or delay infection, PCV2 seroconversion, viremia or both may have been detected if the study by Pujols et al. (2008) had been carried out for a longer period. Because commercial products are known to contain PCV2 DNA, the use of spray-dried plasma on farms does represent a potential risk for PCV2 infection but the risk is likely low in comparison to direct exposure to infected animals or their secretions.

**PCV2 transmission via the aerosol route**

PCV2 transmission via airborne spread has been evaluated; however, to date there is a lack of conclusive evidence that this route results in PCV2 transmission to naïve animals. In a study by Hermann et al. (2008), four 63-day old pigs were inoculated with PCV2 at a dose of $1 \times 10^{4.8}$ TCID$_{50}$/ml. Following infection, oral and nasal swabs, air samples from the pigs, and samples of ambient room air were collected at various intervals for 28 dpi and tested by PCR. Air samples from pigs were collected for 5 min using an impinger and a surgical mask which covered the snout of the pig. PCV2 DNA was not detected in nasal swabs until 14 dpi and only in 3 of 20 samples taken. PCV2 DNA was also not detected from either the aerosol samples taken of the room or directly from the pig. The limit of detection for PCV2 using the impinger detection system is unknown; however, $1 \times 10^{1.1}$ TCID$_{50}$ and $1 \times 10^{1.4}$ TCID$_{50}$
of PRRSV and swine influenza virus were previously detected in a 5 min sampling period (Hermann et al., 2008). No studies have been conducted at the time of this publication which evaluate the spread of PCV2 by aerosol contaminate under field conditions. However, PCV2 DNA has been detected in air samples obtained from swine facilities. Specifically, in a study by Verreault et al., (2009) air samples from 23 commercial swine buildings including multiple finisher facilities, one nursery, and one farrowing building were taken. Each farm was sampled either two or three times, the air samples were evaluated by quantitative real-time PCV2 PCR, and correlated to total dust and bacterial concentrations. While airborne concentrations of PCV2 ranged from $2 \times 10^3$ to $10^7$ genomes per m$^3$ of air, only 40.5% of the virus concentration was explained by the variation in dust concentration and no correlation was found between PCV2 concentrations and airborne bacterial concentrations (Verreault et al., 2009). Additionally, no correlation was found between PCV2 concentration and factors including relative humidity, temperature, CO$_2$ concentration, or animal density, which were evaluated by questionnaire or monitoring devices in the buildings. Interestingly, on three sampling occasions, PCV2 was not detected from the farrowing site indicating the presence of less than 1,389 to 2,778 PCV2 genomic copies per m$^3$ of air (the limit of detection for the sampling apparatus) (Verreault et al., 2009). Because only one farrowing site was sampled and individual animals were not tested for the presence of virus or antibodies, it is unclear whether passive immune protection of piglets resulted in reduced shedding or if this concentration of airborne PCV2 on this site was within a normal farm-to-farm variation. Results of this study indicate that PCV2 DNA can be detected in high concentrations in air samples from commercial swine buildings. However, it is unknown whether the high concentrations of airborne PCV2 DNA were correlated to PCV2 prevalence in the herds.
Additionally, health factors including PCV2 vaccination and the presence of concurrent disease challenges were not analyzed in the study (Verreault et al., 2009). To the author’s knowledge only two studies are available on the topic of aerosol transmission of PCV2 and as only 1/2 studies evaluated PCV2 infection of animals in conjunction with air samples, much more information is needed before it can be determined whether aerosol transmission is a significant risk factor in horizontal transmission of PCV2.

**Potential for transmission of PCV2 through contact of pigs with non-porcine species**

Previous studies have shown that PCV2 can replicate in mice (Cságola et al., 2008; Kiupel et al., 2001; Opriessnig et al., 2009a) and can be transmitted between mice (Cságola et al., 2008) indicating a potential role for mice in the transmission of PCV2. Clinical signs were not observed in mice and microscopic lesions in PCV2-experimentally infected mice were minimal and not similar to those in PCVAD-affected pigs (Kiupel et al., 2005; Opriessnig et al., 2009a). In a recent study by Lőrincz et al. (2010), PCV2 DNA was detected in tissues from 13/20 (65%) of dead mice and 5/21 (23.8%) of dead rats collected from two PCV2-infected swine farms while PCV2 DNA was not detected in tissues from 25 rodents (yellow-necked mice, house mice, common voles) collected in areas not directly connected to PCV2-infected farms (Lőrincz et al., 2010). Limitations of this short communication included a lack of information on the source of control rodents and determining the basis of PCV2-infection status of the herd on detection of PCV2 DNA in 8 or 10 sampled porcine tissues without correlation to data on mortality or clinical PCVAD cases. However, based on the limited information available, indirect transmission of PCV2 secondary to contact with PCV2-infected rodent species is a potential risk factor. Increased information on the
presence of PCV2-infected rodents in association with the PCVAD status of multiple herds is necessary to determine the magnitude of the risk of transmission by this route.

Experimental infection of PCV2 isolates derived from pigs exhibiting PCVAD in cattle (Ellis et al., 2001), sheep (Allan et al., 2000) and rabbits (Quintana et al., 2002) have been unsuccessful as evidenced by the lack of clinical disease, microscopic lesions, PCV2 viremia and seroconversion following infection. Based on the apparent failure of these species to replicate PCV2, evidence to date suggests that it is unlikely that these species pose a risk for transmission of PCV2 to swine. To date no information exists to the author’s knowledge on experimental infection of avian species with PCV2. However, due to lack of detection of PCV2 in species other than swine (Segalés et al., 2005a) and the host specificity of circoviruses within avian species (Todd, 2004), transmission by this route seems unlikely.

To date, there is no serological evidence of PCV2 infection in human, avian, bovine, ovine, caprine, and equine species (Table 3). This data supports the hypothesis that transmission of PCV2 to swine from other species is unlikely. However, PCV2 has been detected by PCR from bovine respiratory and abortion cases (Nayar et al., 1999) and from cases of hemorrhagic diathesis syndrome (Kappe et al., 2010) (Table 3) indicating there remains some risk of cross-species infection. Serological evidence of PCV1 is inconclusive as two studies did not find antibodies against PCV1 in human, bovine, ovine, caprine, murine, avian, and lagomorphs species (Allan et al., 1994; Tischer et al., 1982) while one study reported evidence of exposure to PCV1 in humans, murine and bovine species (Tischer et al., 1995) (Table 3).
In summary, limited information is present on the transmission of PCV2 through indirect routes including the oral and aerosol routes and little (bovine, ovine, lagomorphs, murine species) or no work (avian) has been done to date to directly evaluate transmission of PCV2 between porcine and other species. Of the available information, only the murine species seems to support PCV2 replication making this species a potential for horizontal transmission of PCV2.

**SUMMARY**

It is well documented that PCV2 is a ubiquitous virus based on serological and viremia data from countries worldwide. In addition, PCV2 DNA has been discovered in archived samples prior to the first reports of clinical disease and a shift in PCV2 subtype has occurred worldwide. Following both natural and experimental infection of naïve animals with PCV2, PCV2 DNA has been detected in multiple sample types; however, limited information on the amount of PCV2 present is available and it is unknown whether the detected PCV2 DNA is infectious. Relatively few studies have evaluated the stability of PCV2 in the environment; however, the results indicate that the virus is very resistant to high temperatures and a wide range of pH. In addition, disinfectants have been shown to have variable efficacy against PCV2. Previous studies have documented that transmission of PCV2 occurs by direct or nose-to-nose contact with infected animals and limited evidence to date also suggests that transmission through indirect contact is possible.
REFERENCES


with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. *Veterinary Pathology* **38**: 528-539.


characterization of porcine circovirus type 2 (PCV2) in pigs from Croatia. Research in Veterinary Science 77: 171-175.


Opriessnig T, Patterson, AR, Meng XJ and Halbur PG (2009b). Porcine circovirus type 2 in muscle and bone marrow is infectious and transmissible to naive pigs by oral consumption. *Veterinary Microbiology* **133**: 54-64.


Table 1. Initial seroprevalence of porcine circovirus (PCV) prior to differentiation of PCV1 and PCV2 between 1986-1995.

<table>
<thead>
<tr>
<th>Country</th>
<th>Sample #</th>
<th>Pig type</th>
<th>Location</th>
<th>Farm#</th>
<th>Percentage</th>
<th>Assay</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germany</td>
<td>269</td>
<td>Slaughter animals</td>
<td>Northern Germany</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84%</td>
<td>IFA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1986</td>
<td>Tischer et al.</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Wild boars</td>
<td>Berlin</td>
<td>NA</td>
<td>62%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Breeding animals</td>
<td>Berlin</td>
<td>1</td>
<td>83%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>18</td>
<td>Conventional</td>
<td>NP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>55%</td>
<td>IFA</td>
<td>1989</td>
<td>Dulac and Afshar</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>Cull sows</td>
<td>NP</td>
<td>NA</td>
<td>26%</td>
<td>IFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>SPF research herd</td>
<td>NP</td>
<td>1</td>
<td>0%</td>
<td>IFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>England</td>
<td>87</td>
<td>Porcine serum submitted to a diagnostic laboratory</td>
<td>NP</td>
<td>44</td>
<td>86%</td>
<td>IPMA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1994</td>
<td>Edwards and Sands</td>
</tr>
<tr>
<td>USA</td>
<td>328</td>
<td>Conventional</td>
<td>Georgia</td>
<td>11</td>
<td>51%</td>
<td>IFA</td>
<td>1995</td>
<td>Hines and Lukert</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Conventional</td>
<td>North Carolina</td>
<td>1</td>
<td>60%</td>
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</tr>
<tr>
<td></td>
<td>20</td>
<td>Conventional</td>
<td>Iowa</td>
<td>1</td>
<td>55%</td>
<td>IFA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>NA, not applicable  
<sup>b</sup>IFA, immunofluorescent assay  
<sup>c</sup>NP, not provided  
<sup>d</sup>Timeline of first identification of PCV2 in conventional pigs in various countries as part of retrospective analyses by immunoperoxidase monolayer assay (IPMA), immunofluorescent assay (IFA), immunohistochemistry (IHC), polymerase chain reaction (PCR) or in-situ hybridization (ISH).
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<td>China</td>
<td>Wen et al., 2005</td>
</tr>
<tr>
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<td>Indonesia</td>
<td>Manokaran et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Japan</td>
<td>Onuki et al., 1999</td>
</tr>
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<td></td>
<td>Korea</td>
<td>Choi et al., 2000</td>
</tr>
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<td>Philippines</td>
<td>Maldonado et al., 2004</td>
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<td></td>
<td>Taiwan</td>
<td>Chang et al., 2002</td>
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<td></td>
<td>Thailand</td>
<td>Tantilertcharoen et al., 1999</td>
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<td>Europe</td>
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<td></td>
<td>Belgium</td>
<td>Labarque et al., 2000</td>
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<td>Bulgaria</td>
<td>Motovski et al., 2004</td>
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<td>Croatia</td>
<td>Jemeršič et al., 2004</td>
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<td></td>
<td>Czech Republic</td>
<td>Celera et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Denmark</td>
<td>Allan et al., 1999</td>
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<td></td>
<td>France</td>
<td>LeCann et al., 1997</td>
</tr>
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<td></td>
<td>Germany</td>
<td>Hinrichs et al., 1999</td>
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<td>Greece</td>
<td>Kyriakis et al., 2001</td>
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<tr>
<td></td>
<td>Hungary</td>
<td>Kiss et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Ireland</td>
<td>Spillane et al., 1998; Kennedy et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Netherlands</td>
<td>Wellenberg et al., 2000</td>
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<td></td>
<td>Poland</td>
<td>Podgorska et al., 2009</td>
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<td>Sweden</td>
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<td>Switzerland</td>
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<td>Costa Rica</td>
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<td>Cuba</td>
<td>Peréz et al., 2009</td>
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<td>Mexico</td>
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<td>United States</td>
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<td>Australia</td>
<td>Muhling et al., 2006</td>
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<td>Brazil</td>
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<td>Venezuela</td>
<td>Cano et al., 2005</td>
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<td></td>
<td>Argentina</td>
<td>Machuca et al., 2000; Sarradell et al., 2002</td>
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Table 3: Prevalence of porcine circovirus (PCV) in species other than porcine.

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<tr>
<th>Reference</th>
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<th>Testing method</th>
<th>Sample information</th>
<th>Sample type</th>
<th>Species tested</th>
<th>No. samples positive/Total no. samples tested</th>
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<tbody>
<tr>
<td>Tischer et al.,</td>
<td>PCV1</td>
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<td>Specific information on sample source not provided</td>
<td>Serum</td>
<td>Bovine, murine, human, lagomorph</td>
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<tr>
<td>1982</td>
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<td>Allan et al.,</td>
<td>PCV1</td>
<td>IFA</td>
<td>Specific information on sample source not provided</td>
<td>Serum</td>
<td>Ovine</td>
<td>0/200</td>
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<td>1994</td>
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<td></td>
<td>Bovine</td>
<td>0/350</td>
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<td></td>
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<td>Avian (Turkey, duck and chicken)</td>
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<td>Human</td>
<td>0/200</td>
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<td>Murine</td>
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<td>Lagomorph</td>
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<td>0/150</td>
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<td>Tischer et al.,</td>
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<td>IFA</td>
<td>Healthy persons, blood donors and hospitalized patients</td>
<td>Serum</td>
<td>Human</td>
<td>189/1149</td>
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<td>1995</td>
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<tr>
<td></td>
<td></td>
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<td>Three strains of mice</td>
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<td>Slaughter house serum from 3 different groups of animals</td>
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<td>Bovine abortion case submissions</td>
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<td>Allan et al.,</td>
<td>PCV2</td>
<td>IFA</td>
<td>40 herds</td>
<td>Serum</td>
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<td>2000</td>
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<td></td>
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<td>10 farms (cattle had contact with swine)</td>
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<td></td>
<td></td>
<td></td>
<td>14 flocks</td>
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<td>Ovine</td>
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### Table 3: (Continued)

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<td>Ellis et al., 2000</td>
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<td>cELISA and Whole-cell ELISA</td>
<td>High-risk population of swine veterinarians (n=56) and healthy blood donors (n=33)</td>
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<td>Human</td>
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<td>cELISA IPMA cELISA</td>
<td>Beef and dairy case submissions from 42 farms or feedlots</td>
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<td>Rodríguez-Arrioja et al., 2003a</td>
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<td>Case submissions</td>
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<td>PCR</td>
<td>Case submissions of fatal hemorrhagic diathesis syndrome (HDS)</td>
<td>Tissue</td>
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<td>Non-HDS cases submissions</td>
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<td>Bovine</td>
<td>1/8</td>
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</table>

*Testing methods: IFA = immunofluorescent assay, PCR = polymerase chain reaction, cELISA = competitive enzyme linked immunosorbent assay, ISH = in-situ hybridization, IPMA = immunoperoxidase monolayer assay, IHC = immunohistochemistry*
**Fig. 1.** Timeline of first identification of PCV2 in conventional pigs in various countries as part of retrospective analyses by immunoperoxidase monolayer assay (IPMA), immunofluorescent assay (IFA), immunohistochemistry (IHC), polymerase chain reaction (PCR) or in-situ hybridization (ISH).

- **United Kingdom:** PCV2 DNA detected by PCR from DNA extracted from archived paraffin wax-embedded tissue blocks (Grierson *et al.*, 2004)
- **Belgium:** PCV2 detected by IMPA in serum collected from sows in slaughter houses (Sanchez *et al.*, 2001)
- **Canada:** PCV2 detected by IFA on serum samples collected from slaughterhouse sows (Magar *et al.*, 2000b)
- **Mexico:** PCV2 detected by IPMA from serum samples (Ramírez-Mendoza *et al.*, 2009)
- **Northern Ireland:** PCV2 detected by competitive ELISA on serum samples collected from commercial herds (Walker *et al.*, 2000)
- **Spain:** PCV2 DNA detected by ISH in archived formalin-fixed, paraffin-embedded tissues (Rodríguez-Arrioja *et al.*, 2003b)
- **Northern Germany:** PCV2 DNA detected by ISH and PCR from pig tissues (Jacobsen *et al.*, 2009)
- **Switzerland:** PCV2 antigen detected by IHC on paraffin-embedded lymphoid tissues from pigs (Staebler *et al.*, 2005)
- **Thailand:** PCV2 DNA detected by PCR in paraffin-embedded tissues (Kiatipattanasakul-Banlunara *et al.*, 2002)
- **Brazil:** PCV2 DNA and antigen detected by PCR and IHC in formalin-fixed, paraffin-embedded tissues from pigs (Ciacci-Zanella *et al.*, 2006)
- **Japan:** PCV2 DNA detected by PCR on formalin-fixed, paraffin-embedded lymph nodes (Hasegawa *et al.*, 2001)

**Timeline:**
- 1962
- 1969
- 1970
- 1973
- 1985
- 1986
- 1988
- 1993
CHAPTER 3. Comparison of three enzyme-linked immunosorbent assays to detect porcine circovirus-2 (PCV-2)-specific antibodies after vaccination or inoculation of pigs with distinct PCV-1 or PCV-2 isolates

A paper published in the Journal of Veterinary Diagnostic Investigation

Abby R. Patterson, John Johnson, Sheela Ramamoorthy, Xiang-Jin Meng, Patrick G. Halbur, Tanja Opriessnig

ABSTRACT

Porcine circovirus type 2 (PCV2) serology is frequently used to determine PCV2 status and optimal timing of PCV2 vaccination in the field. The objectives of the current study were to determine the diagnostic accuracy of 3 currently available commercial anti-immunoglobulin G (IgG)–PCV2 enzyme-linked immunosorbent assays (ELISAs) and to compare the ability of the 3 assays to detect and differentiate between anti–PCV2a and anti–PCV2b antibodies as well as anti–PCV2 and anti–PCV1 antibodies. Fifty-five, 3-week-old, conventional pigs were randomly allocated to 7 groups: 1) negative controls (n = 7); 2) PCV2a (n = 8; inoculated with PCV2 ISU-40895); 3) PCV2b (n = 8; inoculated with PCV2 NC-16845); 4) PCV1 (n = 8); 5) vaccine A (n = 8; Ingelvac® CircoFLEX™); 6) vaccine B (n = 8; Circumvent® PCV2); and 7) vaccine C (n = 8; Suvaxyn® PCV2 One Dose). Blood samples were collected weekly, and all sera were tested by 3 different anti–PCV2–IgG ELISAs. The results indicated that all ELISAs had area under the receiver operating curve values greater than 0.94, detected both anti–PCV2a and -2b antibodies with no differentiation, and did not detect anti–PCV1 antibodies in infected animals. One of the
ELISAs was able to distinguish pigs vaccinated with vaccine B from pigs inoculated with either PCV2a or PCV2b.

**Key words:** Antibodies; enzyme-linked immunosorbent assay; *Porcine circovirus* type 2; serology; vaccination.

**INTRODUCTION**

Porcine circovirus (PCV; family *Circoviridae*; genus Circovirus) was first identified as a contaminant of the permanent porcine kidney cell line PK-15 in 1974. The virus is nonenveloped and comprised of a single-stranded, circular DNA genome. Two major types of PCV have been identified, PCV type 1 (PCV1) and type 2 (PCV2). PCV1 has been shown to be nonpathogenic in pigs under experimental conditions. In contrast, infection with PCV2 has been associated with respiratory disease, enteritis, reproductive failure, porcine dermatitis and nephropathy syndrome (PDNS), and systemic infections, which are known as porcine circovirus associated disease or PCVAD. While the pathogenesis of PCVAD remains unclear, it can result in high levels of PCV2 viremia in 5–30% of a swine population. In affected animals, mortality can reach 70–80%.

Based on sequence analyses of PCV2, type 2 can be further divided into 2 main phylogenetic clusters, PCV2a and PCV2b. The PCV2 genome is comprised of 2 known open reading frames (ORFs) that code for functional proteins. The ORF1 gene encodes for a replication-associated protein (Rep), which is essential for virus replication. The ORF2 gene
encodes for the capsid protein, which has been shown to be immunogenic \(^{20,21}\) and which has a greater nucleotide variation than ORF1.\(^{15}\)

Several commercial PCV2 vaccines became available in North America in 2006. As of January 2008, 3 products are fully licensed and available in the United States for use in healthy pigs. Two of the vaccines contain the PCV2 capsid protein expressed in a baculovirus (BA) system as an antigen\(^ {6,8}\) The third vaccine is comprised of an inactivated PCV1–2 chimera virus.\(^ {7}\) Other notable differences between the 3 vaccines include the dose and timing of administration: 2-ml versus 1-ml injections, and one time administration versus booster vaccination.\(^ {22}\)

The release of PCV2 vaccines provides an impetus for assessing the PCV2 status of herds in order to determine the best time for vaccination. One method of assessing when and if PCV2 is circulating in a herd is to conduct a cross-sectional serological survey to determine the prevalence and levels of anti–PCV2 antibodies over time using one or more of the serological tests for PCV2 including various ELISAs.\(^ {3,16,20,25,29,30}\) Most ELISA tests use the ORF2 region as antigen since it is specific and immunogenic for PCV2.\(^ {28}\) Although most assays are based on PCV2 ORF2, the methods of antigen production differ among assays. Specifically, antigen has been produced by expression of the capsid protein in a BA vector system,\(^ {3,16,20}\) in live virus preparations in PK-15 cells,\(^ {29}\) or through expression in \textit{Escherichia coli}.\(^ {25,30}\) While reports indicate that ELISAs based on the ORF2 region of PCV2 are useful, a direct comparison of the diagnostic accuracy of currently available ELISAs has not yet been performed to the authors’ knowledge. Additionally, reports on the ability of the assays to detect and differentiate between anti–PCV2a, anti–PCV2b, or vaccine-induced antibodies are lacking.
To address the gaps in knowledge on current PCV2 ELISAs, the current study had the following objectives: (1) to determine the diagnostic accuracy of 3 commonly used ELISAs, (2) to determine whether each assay can detect and/or differentiate between anti–PCV2a and anti–PCV2b antibodies, and (3) to determine whether PCV2–based ELISAs can detect anti–PCV1 antibodies.

MATERIALS AND METHODS

Animals and housing

Animals were purchased from a PCV2–negative herd, weaned at 2 weeks of age, and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University (Ames, IA). On the day of delivery, the pigs were randomly assigned to 1 of 7 rooms, each containing a 1.2 × 2.4 m raised wire deck pen equipped with 1 nipple drinker and 1 self-feeder.

Experimental design

Three-week-old, conventional pigs were randomly allocated to 7 groups and inoculated with either PCV1, PCV2a, or PCV2b, vaccinated with commercially available PCV2 vaccines, or left as negative controls as follows: 1) negative controls (n = 7); 2) PCV2a (n = 8); 3) PCV2b (n = 8); 4) PCV1 (n = 8); 5) vaccine A (n = 8); 6) vaccine B (n = 8); and 7) vaccine C (n = 8). Upon arrival, pigs were bled and serum was tested by quantitative real-time PCV2 polymerase chain reaction (PCR)\textsuperscript{23} to detect PCV2 DNA and by a PCV2 fluorescent antibody test (FAT)\textsuperscript{24} to detect anti-PCV2 antibody. The experimental
inoculations and vaccinations were performed on trial day 0 at which time the pigs were 3 weeks of age. All pigs were bled at weekly intervals until trial day 49. All serum samples were tested by 3 different ELISAs, and results were compared. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee.

Inoculation

On trial day 0, pigs in the PCV2a, PCV2b, and PCV1 groups were inoculated with 2 ml intramuscularly (IM) and 3 ml intranasally of $10^{4.5}$ 50% tissue culture infective dose (TCID$_{50}$)/ml of the appropriate virus inoculum. Animals were inoculated with the following vaccines: PCV2a group (ISU-40895; GenBank accession number AF264042); PCV2b group (NC-16845; GenBank accession number EU340258); and PCV1 group (well-characterized PCV1 clone). To confirm that the pigs in the PCV1 group were infected with PCV1, serum samples were analyzed by PCR for PCV1 DNA. To confirm that PCV2–inoculated pigs were infected, serum samples from all pigs were tested by PCR for PCV2 DNA. Additionally, a PCR product recovered on trial day 49 from a PCV2a– and a PCV2b–inoculated pig was sequenced and compared to the respective inoculum.

Vaccination

Pigs in vaccine groups A, B, and C were each vaccinated with the respective vaccines according to the recommendations of the manufacturers. Briefly, animals in the vaccine A group (Ingelvac® CircoFLEX™) received a 1-ml IM injection on trial day 0. Pigs in the vaccine B group (Circumvent® PCVb) received a 2-ml IM injection on day 0 and a second 2-ml IM injection on day 14. Pigs in the vaccine C group (Suvaxyn® PCV2 One Dosec) received a 2-ml IM injection on trial day 0.

Serology
All serum samples collected from the pigs over time were tested by a previously described indirect PCV2 FAT and each of the following 3 ELISAs:

**ELISA 1.** This in-house, modified, indirect PCV2 ELISA was performed as previously described. For this assay, alternating rows on the ELISA plate were coated with an ORF2 antigen (expressed in a BA expression vector) or a BA antigen (BA expression vector without the PCV2a ORF2 insert). The PCV2 isolate used for antigen production, ISU-31 (GenBank accession number AJ223185) had 99% similarity to the PCV2a isolate used for inoculation. The following modifications were made to the previously described protocol: following incubation, the plates were washed 5 times with 0.1 M of phosphate buffered saline (PBS; pH 7.2) and 0.1% Tween 20 using a microplate washer. For visualization of results, 100 µl of 3,3’,5,5’-tetramethylbenzidine was added to the wells. Following a 15-min incubation at 25°C, the reaction was stopped by adding 50 µl of 1 M H₂SO₄. Results were reported as sample-to-positive (S/P) ratios. All samples were run in duplicate.

Initially, a corrected optical density (OD) value was calculated for the 2 replicates of each sample and the positive control: 
\[
\frac{\text{ORF2 OD} + \text{ORF2 OD}}{2} - \frac{\text{BA OD} + \text{BA OD}}{2},
\]
where ORF2 is ORF2 antigen, and OD is optical density value at 450 nm. To generate the S/P ratio, the sample-corrected OD was divided by the positive control–corrected OD; S/P ratios <0.2 were considered negative, and S/P ratios ≥0.2 were considered positive. The previously determined diagnostic sensitivity and specificity of this method using a 0.2 cut-off were 79.9% and 99.6%, respectively.

**ELISA 2.** This commercially available competitive ELISA (Serelisa® PCV2 Ab Mono Blocking) was preformed according to manufacturer’s recommendations. The plates provided by the manufacturer were coated with purified PCV2 antigen. Results were reported
as an OD ratio of the sample OD value to the negative control OD value. A plate-specific cut-off was used such that samples with OD ratios ≤0.15 were considered positive, samples with an OD ratio of 0.15–0.20 were considered suspect, and samples with an OD ratio ≥0.20 were considered negative as recommended by the manufacturer.

*ELISA 3.* This commercially available capture PCV2 ELISA (Ingezim Circovirus IgG) was also performed according to manufacturer’s recommendations. The plates provided in the assay came coated with monoclonal antibodies specific for swine IgG. The antigen provided in the assay was a recombinant PCV2b protein produced through expression in a BA vector (Jacques Delbecque, personal communication, 3/5/08). Results were reported as an OD ratio of the sample OD value to the positive-control OD value. The plate-specific cut-off was based on the OD 450 nm value of the positive control × 0.3 as recommended by the manufacturer.

**Statistics**

Diagnostic accuracy of each assay was evaluated using receiver operating characteristic (ROC) analysis. This method results in area under the ROC curve (AUC) values ranging from 0.5 to 1, where 0.5 indicates the test is not discriminating between positive and negative animals and 1 indicates perfect discrimination.\(^\text{31}\) Area under the ROC values were compared pairwise between tests using a previously described method.\(^\text{11}\) All ROC calculations were performed on continuous data with infection status as the indicator of true infection status using MedCalc.\(^\text{1}\) Summary statistics were calculated for all groups to assess the overall quality of the data including normality. Continuous data was analyzed using multivariate analysis of variance (MANOVA). If a significant (\(P < 0.05\)) change over time was noted for the outcome variable, a nonparametric Kruskal-Wallis 1-way analysis of
variance (ANOVA) was performed at each time point. If this nonparametric ANOVA test was significant ($P < 0.05$), then pairwise Wilcoxon tests were used to assess differences between groups. Statistical analysis was performed using JMP software.

RESULTS

Diagnostic accuracy of assays

All assays had AUC values greater than or equal to 0.94 for all trial days using ROC generated optimal cut-offs; no statistical differences were noted among the assays (Table 1). When data was dichotomized using the manufacturer’s recommended cut-off values (suspect samples were considered negative), the sensitivity of all 3 assays was substantially reduced at trial day 14 (Table 2). In contrast, the specificity of ELISAs 2 and 3 was improved. When the manufacturer’s recommended cut-off was utilized at trial day 49, the sensitivity of ELISAs 2 and 3 was substantially reduced (Table 2). The number of pigs classified as positive or negative based on a sample-to-positive ratios of 0.2 (ELISA 1) or the manufacturer’s recommendations (ELISA 2 and 3) by the 3 ELISAs at trial days 14 and 49 is presented in Table 3.

Detection of PCV2a and PCV2b

Infection status for PCV2a and PCV2b groups was confirmed by PCR and sequence analysis (Opriessnig T, Ramamoorthy S, Madson DM, et al.: 2007, Experimental comparison of the virulence of PCV2a and PCV2b isolates in a conventional pig model. Proc Conf Res Workers Anim Dis 88:145). To assess whether PCV2a could be differentiated from PCV2b on any of the 3 ELISAs, the following null hypothesis was tested: there is no difference between PCV2a and PCV2b ELISA S/P or OD values on ELISAs 1, 2, or 3. Statistical
analysis using a Wilks’ lambda multivariate test indicated a significant ($P < 0.0001$) difference over time between control, PCV2a and PCV2b groups. A nonparametric ANOVA at each time point revealed a significant difference between negative controls, PCV2a and PCV2b groups ($P < 0.05$) for all tests. Further analysis using pairwise Wilcoxon tests indicated that no significant differences were noted between PCV2a and PCV2b groups on any of the assays (Fig. 1).

Detection of PCV1

Serum samples from pigs in the PCV1 group were positive for PCV1 DNA by PCR starting on trial day 7 (data not shown). No positive reactions were detected in serum samples from PCV1–inoculated animals on any of the 3 ELISAs at any time during the trial (data not shown).

Detection of antibodies in vaccinated animals

Mean and standard error S/P and OD ratios for each group are provided in Figure 2. Results from ELISA 1 indicated that animals in the vaccine A group had significantly lower mean S/P ratios in comparison to PCV2a– and PCV2b–inoculated animals from trial days 14–49 ($P < 0.0005$ for all days; Fig. 2a). Animals in the vaccine B group also had significantly lower mean S/P ratios from trial days 28–49 ($P < 0.03$ for all days) in comparison to PCV2–inoculated animals. In contrast, animals in the vaccine C group had a significantly ($P = 0.01$) higher mean S/P ratio at trial day 28 in comparison to PCV2–inoculated animals (Fig. 2a). In contrast to ELISA 1, no significant difference was noted between animals in either the vaccine A or B group and the PCV2–inoculated animals based on ELISA 2 ($P > 0.05$ for all days; Fig. 2b). Animals in the vaccine C group had a significantly lower (more positive) OD ratio than that in challenged animals at trial day 28.
and 35 ($P < 0.008$ for both days; Fig. 2b). Results from ELISA 3 were similar to ELISA 1 (Fig. 2c); animals in the vaccine A group had significantly lower mean OD ratios in comparison to PCV2–inoculated animals at trial days 14–49 ($P < 0.0006$ for all days).

Following comparative analysis of the S/P values of vaccinated and PCV2–inoculated animals on ELISA 1, an S/P ratio decrease in the vaccine B group from trial days 21–28 was noted. Further analysis at trial day 21 revealed that in comparison to PCV2a– and PCV2b–inoculated pigs, the OD values of the wells containing BA antigen were significantly higher ($P = 0.0006$) in the vaccine B group. This significant increase in OD values of the BA wells for the vaccine B group was apparent through the end of the trial ($P < 0.0001$ for trial days 28–49). The effect of an increase in OD values in the BA wells on the S/P ratio is illustrated graphically in Figure 3. Optical density values of the BA wells for all groups except the vaccine B group were indistinguishable from background levels (data not shown). A similar increase in OD values of the BA wells was not noted on any trial days in the vaccine A or C group or with ELISA 2 and 3.

**DISCUSSION**

Receiver operating characteristic analysis has become an increasingly valuable tool for comparison of diagnostic tests.\(^9\,32\) Unlike the kappa statistic, ROC analysis determines the sensitivity and specificity of the test based on classification of true status (infected vs. negative controls in the current study) at multiple cut-offs by utilizing the complete set of continuous data.\(^9\) Interestingly, when the 3 ELISAs were compared by ROC analysis, AUC values were very high (>0.9). This indicated that all tests were able to distinguish between
negative and positive animals using ROC analysis generated optimal cut-offs (cut-offs that provide the highest sensitivity and specificity) based on continuous data from the experiment. In contrast, when the manufacturer’s cut-off values were used, sensitivity was substantially reduced. While the current study was limited by a small sample size, ROC analysis data suggests that altering the cut-off value will increase the sensitivity of ELISAs 2 and 3. Further studies with larger sample sizes are necessary to determine an appropriate cut-off. The manufacturers’ sensitivity and specificity for ELISAs 2 and 3 at the recommended cut-offs were not indicated on the package insert and could not be compared to the results in the present study.

As the majority of herds worldwide are seropositive for PCV2, ELISAs are most commonly used in cross-sectional analyses to target optimal timing of vaccination. Recent trends indicate an increased incidence of detection of PCV2b isolates and the association of these strains with high mortality in herds with increased incidence of severe mortality in swine populations.\(^5,\,12\) This has stimulated interest in the use of serological tests to monitor PCV2b. As such, there is a need to know whether commonly used ELISAs are detecting both anti–PCV2a and anti–PCV2b antibodies. Results of the current study indicate that all 3 ELISAs can detect both antibodies but cannot differentiate between anti–PCV2a and anti–PCV2b antibodies. This was independent of the origin of the coating antigen used since at least one assay used PCV2a (ELISA 1) and one assay used PCV2b (ELISA 3) for antigen production.

Nucleotide sequence homology between PCV1 and PCV2 ranges from 69% to 79.5%.\(^{10,\,18,\,19}\) A previous report indicated that there was no serological cross-reactivity between the ORF2 region of PCV2 and PCV1,\(^{17}\) or between PCV1 and PCV2 polyclonal
antisera and monoclonal antibodies using an indirect FAT. Conversely, ORF1 regions of PCV2 do exhibit serological cross-reactivity with PCV1. Results of the current study confirm previous reports and indicate that PCV1–inoculated animals were not detected with any of the ELISAs evaluated. Although the PCV1–inoculated animals were found to be PCV1–positive by PCR, an anti–PCV1 ELISA assay was not available to confirm the presence of anti–PCV1 antibodies in these animals.

Following the introduction of vaccines into the U.S. market in 2006, the prevalence of seropositive animals has grown steadily. Therefore, an assay able to differentiate vaccinated and naturally infected animals would provide a useful tool for veterinary practitioners in situations where they are assessing vaccination protocol compliance issues. Although distinguishing vaccinated animals on the basis of either an S/P or OD ratio would not be possible based on the results of the current study, using the OD value of the BA well on ELISA 1 may provide a means of differentiation for vaccine B. In contrast to the other 2 ELISAs, ELISA 1 utilizes BA derived from a baculovirus expression vector that does not contain the PCV2 ORF2 insert to control for nonspecific background binding. Serum samples from both vaccine A and B group pigs were expected to have increased OD values for the BA wells as both products utilize the baculovirus expression vector for antigen production. Interestingly, only the serum samples from the vaccine B group showed a significant increase in OD value in the BA well. As the increase in OD value of the BA well occurred following the administration of the second dose of vaccine B, it can be speculated that 2 doses of vaccine A may lead to the same response as seen with vaccine B. Alternatively, differences in vaccine preparation or type of adjuvant between the 2 products
may account for the lack of cross-reactivity in BA well OD values of animals in the vaccine A group.

While providing a means of differentiating vaccinated and naturally infected animals, significant increases in OD values alone should be interpreted carefully as results could be confounded by vaccination with other products in which the baculovirus vector is utilized, serum factors other than antibodies against BA, or other unknown nonspecific reactions. These nonspecific reactions could falsely elevate the OD value. As an S/P ratio is not generated when looking at the individual OD value from the BA well, no control for this is in place with the current assay.

The lack of increase of the S/P ratio in ELISA 1 for vaccine B group cannot be interpreted as a lack of seroconversion because a high OD value for the BA could be lowering the S/P ratio. Therefore, the OD value for the BA well should be observed in addition to the S/P ratio or one of the other 2 assays should be utilized to assess seroconversion with this product. Reasons for the decreased average OD on ELISA 3 for the vaccine A group may include vaccine preparation, type of adjuvant, or dose of administration. It should be noted that while antibody levels following vaccination were determined in the current study, there was no challenge of animals. Therefore, the ability of vaccination to protect against disease was not evaluated in this trial.

ACKNOWLEDGEMENTS

The authors wish to thank Paul Thomas for assistance with animal work, and the staff at Iowa State University Veterinary Diagnostic Laboratory for assistance with serology work.
This study was founded by the PCV2 research award, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO.

**SOURCES AND MANUFACTURERS**


b. Intervet Inc., Millsboro, DE.

c. Fort Dodge Animal Health, Fort Dodge, IA.


e. Sigma-Aldrich, St. Louis, MO.

f. Kirkegaard & Perry Laboratories, Gaithersburg, MD.

g. Synbiotics Europe SAS, Lyon, France.

h. Ingenasa, Madrid, Spain.

i. MedCalc Software version 9.1.0.1, Mariakerke, Belgium.

j. JMP® version 6.0.0, SAS Institute Inc., Cary, NC.

**REFERENCES**


**Table 1.** Area under the receiver operating curve (AUC) values (±standard error) for each of the 3 enzyme-linked immunosorbent assays (ELISAs) on different trial days.*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Trial day</th>
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<tbody>
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<td></td>
<td></td>
<td>14</td>
<td>21</td>
<td>28</td>
<td>35</td>
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<td>ELISA 1</td>
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<td>0.99±0.01</td>
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<tr>
<td>ELISA 2</td>
<td>0.97±0.03</td>
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<td>1.00±0.00</td>
<td>1.00±0.00</td>
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<tr>
<td>ELISA 3</td>
<td>0.96±0.03</td>
<td>0.99±0.01</td>
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<td>1.00±0.00</td>
<td>0.99±0.01</td>
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</table>

* Receiver operating characteristic analysis was performed by denoting *Porcine circovirus 2* subtype a (PCV2a), PCV2b, vaccine A, vaccine B, and vaccine C pigs as “true positive” (n = 40) and negative controls and PCV1 as “true negative” animals (n = 15). AUC values range from 0.5 to 1, where 1 indicates the diagnostic test would perfectly discriminate between PCV2–positive and –negative pigs when using a given sample, and 0.5 indicates there is no discrimination between groups.
Table 2. Comparison of sensitivity and specificity of each of the 3 enzyme-linked immunosorbent assays (ELISAs) at trial days 14 and 49 using various cut-offs for data dichotomization.

<table>
<thead>
<tr>
<th>Assay</th>
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<td></td>
<td>Cut-off</td>
<td>Sensitivity (%)</td>
<td>Specificity (%)</td>
<td>Cut-off</td>
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<td></td>
</tr>
<tr>
<td>ELISA 1</td>
<td>0.03*</td>
<td>87.5</td>
<td>100</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>0.20†</td>
<td>45</td>
<td>100</td>
<td>0.20</td>
</tr>
<tr>
<td>ELISA 2</td>
<td>0.52*</td>
<td>97.5</td>
<td>93.3</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.15‡</td>
<td>22.5</td>
<td>100</td>
<td>0.15</td>
</tr>
<tr>
<td>ELISA 3</td>
<td>0.10*</td>
<td>85</td>
<td>93.3</td>
<td>0.15</td>
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<td></td>
<td>0.45§</td>
<td>30</td>
<td>100</td>
<td>0.45</td>
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</table>

* Cut-off determined by receiver operating characteristic (ROC) analysis.
† Cut-off determined from previous in-house data on test performance.
‡ Cut-off determined by generating an optical density (OD) ratio by dividing the average sample OD value by the average OD value of the negative control well; an OD ratio ≤0.15 was considered positive as recommended by the manufacturer.
§ Average plate cut-off. Individual plate cut-offs were determined by multiplying the average OD value of the positive control well by 0.3 as recommended by the manufacturer.
Table 3. Number of pigs classified as positive (+) or negative (–) by the 3 enzyme-linked immunosorbent assays (ELISAs) and a PCV2 fluorescent antibody test (FAT) at trial days 14 and 49.*

<table>
<thead>
<tr>
<th>Assay</th>
<th>Trial day 14</th>
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<th>Trial day 49</th>
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<tr>
<td></td>
<td>ELISA 2 (+)</td>
<td>ELISA 2 (–)</td>
<td>FAT (+)</td>
<td>ELISA 2 (+)</td>
<td>FAT (+)</td>
<td>ELISA 3 (+)</td>
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<tr>
<td></td>
<td>ELISA 2 (–)</td>
<td></td>
<td>FAT (–)</td>
<td>ELISA 3 (–)</td>
<td></td>
<td></td>
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<tr>
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<td>0 (15)</td>
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<td></td>
<td>0 (34)</td>
<td></td>
<td></td>
<td>0 (6)</td>
<td>0 (15)</td>
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<tr>
<td>ELISA 3</td>
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<td></td>
<td>22 (12)</td>
<td>34 (6)</td>
<td>0 (15)</td>
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<td></td>
<td>9 (22)</td>
<td></td>
<td></td>
<td>1 (20)</td>
<td>0 (15)</td>
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</tr>
<tr>
<td>FAT</td>
<td>8 (25)</td>
<td></td>
<td>0 (22)</td>
<td>22 (12)</td>
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<td></td>
<td>7 (25)</td>
<td></td>
<td>0 (22)</td>
<td>1 (20)</td>
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</table>

*For ELISA 1, data was dichotomized such that sample-to-positive ratios ≥0.2 were considered positive. For ELISAs 2 and 3, data was dichotomized as recommended by manufacturer. Suspects were considered to be negative. For the FAT, data was dichotomized such that titers ≥ 1:20 were considered positive.
Figure 1. Mean sample-to-positive (S/P) ratio and standard error for enzyme-linked immunosorbent assay (ELISA) 1 (a), and mean optical density (OD) ratio and standard error for ELISA 2 (b) and ELISA 3 (c). Controls (n = 7; negative controls), *Porcine circovirus* type 2 subtype a (PCV2a; n = 8; inoculated with PCV2 ISU 40895), and PCV2b (n = 8; inoculated with PCV2 NC 16845).
Figure 2. Mean sample-to-positive (S/P) ratio for enzyme-linked immunosorbent assay (ELISA) 1 (a), and mean optical density (OD) ratio and standard error for ELISA 2 (b) and ELISA 3 (c). Negative controls (n = 7), *Porcine circovirus* type 2 (PCV2; n = 16; combined PCV2a and PCV2b groups), vaccine A (n = 8), vaccine B (n = 8), and vaccine C (n = 8).
Figure 3. Mean and standard error sample to positive (S/P) ratio and average optical density for the baculovirus (BA) antigen and open reading frame 2 (ORF2) wells on enzyme-linked immunosorbent assay (ELISA) 1. All values are for animals in the vaccine B (n = 8) group.
CHAPTER 4. Shedding and infection dynamics of porcine circovirus type 2 (PCV2) in pigs naturally or experimentally infected

A paper submitted to *Veterinary Microbiology*

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**ABSTRACT**

The objective of this study was to determine the amount and infectivity of porcine circovirus type 2 (PCV2) shed in nasal, oral and fecal secretions following natural or experimental infection. Fecal, oral and nasal swabs and serum were collected at regular intervals until 209 days post-farrowing (DPF) from four PCV2-naturally infected pigs (Trial 1) or until 69 days post-inoculation (DPI) from five PCV2-experimentally inoculated pigs (Trial 2). To assess the infectivity of the PCV2 present in excretions, secretions, and on a hypodermic needle, 28 PCV2-naïve pigs were inoculated with various samples obtained from Trial 2 pigs (Trial 3). In Trial 1 and 2, PCV2 DNA was detected in all sample types. There were no differences in the onset of virus shedding (Trial 2) or in the amount of PCV2 DNA present in different sample types over time (Trial 1 or Trial 2). PCV2 DNA was detectable in sera and secretions in pigs at 209 DPF (Trial 1) and at 69 DPI (Trial 2). Intraperitoneal inoculation with contaminated fecal, nasal and oral samples; intranasal inoculation of nasal secretions; and feces fed to naïve animals resulted in viremia and seroconversion (Trial 3). Viremia and microscopic lesions were noted in one animal injected using a contaminated
needle (Trial 3). Natural or experimental PCV2 exposure results in a long term infection.

PCV2 is shed in similar amounts by nasal, oral and fecal routes. The detected PCV2 DNA in Trial 2 was infectious to naïve pigs and therefore multiple routes are likely important in PCV2 transmission.

Key words: Epidemiology, infectivity, Porcine circovirus type 2, PCV2, shedding, transmission

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, single-stranded DNA virus which emerged in the 1990’s as an economically important swine pathogen (Harding et al., 1998). While the majority of the swine population worldwide is seropositive for PCV2, severe clinical disease manifest as respiratory, reproductive, enteric or systemic disease occurs only in 5 to 30% of infected populations (Opriessnig et al., 2007). Based on the sequence analyses of PCV2 strains, isolates can be divided into five genotypes (PCV2a-e). PCV2a and PCV2b have been documented worldwide (Segalés et al., 2008), PCV2c has been documented only in archived samples from Denmark (Dupont et al., 2008), and PCV2d and PCV2e have only been identified in China to date (Wang et al., 2009).

Molecular diagnostic tools have been used to better understand PCV2 transmission by detecting and quantifying PCV2 DNA in various excretions and tissues (Segalés et al., 2005b) from naturally infected pigs with various clinical presentations. The length of PCV2 viremia and onset of seroconversion in naturally-infected populations has also been investigated from 6 through 25 weeks of age (Carasova et al., 2007) and the authors found
that anti-PCV2 IgM appeared first at 8 weeks of age followed by anti-PCV2 IgG which appeared at 10 weeks of age. PCV2 viral load also peaked at 10 weeks of age but PCV2 viremia was present until termination of the study (Carasova et al., 2007). A longitudinal case-control study beginning at 1 week of age and extending through 8-21 weeks of age was conducted on farms naturally-affected by porcine circovirus associated disease (PCVAD) in Denmark and Spain (Grau-Roma et al., 2009) and the authors found an increasing PCV2 load parallel to waning of passively-acquired antibody levels. The highest PCV2 loads correlated with clinical signs of disease and clinically-affected pigs had a higher PCV2 prevalence and higher PCV2 loads compared to unaffected pigs. Moreover, the amount of PCV2 DNA present in sera correlated well with the amount present in nasal and fecal swabs in these pigs (Grau-Roma et al., 2009). However, results from these observational studies of naturally-infected pigs may be influenced by pathogen and environmental co-factors that are known to increase the duration and level of PCV2 replication (Dorr et al., 2007).

Detection of PCV2 DNA in various samples following experimental PCV2-infection has been reported (Bolin et al., 2001; Caprioli et al., 2006; Krakowka et al., 2000; Shibata et al., 2003); however, little is known about the amount of PCV2 DNA shed within secretions or whether the PCV2 DNA detected by PCR remains infectious. In one study, 5-week-old colostrum-deprived (CD) pigs were oronasally inoculated with PCV2 and PCV2 DNA was detected in fecal and tonsillar swabs from 1 to 21 days post-inoculation; however, PCV2 DNA was not detected in serum, plasma or whole blood until 9 DPI (Caprioli et al., 2006). Similarly, PCV2 DNA was detected in oropharyngeal and nasal swabs, feces and sera in CD pigs intranasally-inoculated with PCV2 from 1 to 70 DPI (Shibata et al., 2003). In another study, in order to determine tissue distribution of PCV2 DNA and duration of viral
persistence, cesarean-derived, colostrum-deprived (CDCD) pigs were intranasally and subcutaneously inoculated with PCV2. PCV2 DNA was detected by PCR from all tissues examined including brain, lymphoid tissue, bone marrow, kidney, ileum, liver, heart, lung, spleen, thymus, tonsil and pancreas on 20 and 28 DPI (Bolin et al., 2001). The length of PCV2 persistence in different organs varied; however, at 125 DPI, virus was only detected in spleen and distal ileum (Bolin et al., 2001).

With numerous routes of virus shedding and persistence of virus in multiple tissues, both direct contact with infected animals and indirect contact through exposure to secretions or contaminated fomites are likely routes of PCV2 transmission. Naïve contact pigs exposed to experimentally-infected pigs at 42 DPI seroconverted 21 days post-exposure indicating that the experimentally-infected pigs remained infectious until at least 42 DPI (Bolin et al., 2001). Similarly, PCV2-naïve contact animals were comingled with 5-to-9 week old SPF or conventional pigs immediately following experimental PCV2 inoculation and 90% of the contact animals (10 of 11 pigs) developed clinical PCVAD (Albina et al., 2001). When experimentally-PCV2-infected pigs were co-mingled with naïve contact pigs (within pen) or placed in a separate pen in the same room (between-pen), transmission (as evidenced by viremia and seroconversion) occurred most efficiently when naïve animals were mixed with infected pigs between 11 and 18 days post-infection. Comingling at 25, 32 and 39 days post infection resulted in fewer primary infections indicating decreased transmission (Andraud et al., 2008).

To our knowledge, a detailed investigation of the infection dynamics in segregated early weaned, PCV2-positive or naïve pigs experimentally-infected with PCV2 without coinfection with porcine reproductive and respiratory virus (PRRSV), swine influenza virus...
(SIV) or parovirus (PPV) has not been reported to date. The objectives of this study were to determine and compare the amount of PCV2 shedding in nasal, oral and fecal secretions following natural (Trial 1) or experimental (Trial 2) PCV2 infection and to determine the infectivity of the PCV2 DNA detected in excretions, secretions or on the surface of a mechanical vector (needle) (Trial 3).

MATERIALS AND METHODS

2.1 Experimental Design

The experimental protocol for all studies was approved by the Iowa State University Institutional Animal Care and Use Committee (study #10-07-6441-S). The experimental design is summarized in Table 1. The pigs used for experimental inoculation were purchased from different herds routinely tested for major swine pathogens and known to be free of PRRSV, SIV and PPV. The pigs were weaned at three weeks of age and transported to a BSL-2 animal holding facility at Iowa State University. All pigs were housed in separate rooms by trial and groups, respectively, with a solid concrete floor, a separate ventilation system, and one nipple drinker. The pigs were fed with a balanced, pelleted, complete feed ration free of animal proteins and antibiotics (Nature’s Made, Heartland Coop) once a day. All samples collected during the experiments were tested for the presence of PCV2 DNA and anti-PCV2-antibodies. At the termination of each trial, the animals were humanely euthanized.

2.1.1. Trial 1 (Naturally PCV2-infected pigs)

At arrival in the research facility, the pigs for Trial 1 (n=4) were PCV2 positive as evidenced by the presence of PCV2 DNA and anti-PCV2-antibodies in serum at 13 DPF. The
pigs originated from 4 litters. The litters were selected based on high amounts of PCV2 DNA in serum at 13 DPF. The dams of these pigs were also PCV2 positive (DNA and antibodies; data not shown); horizontal transmission cannot be confirmed or ruled out as the piglets were not tested immediately after birth. Sequence analysis of the open-reading frame 2 (ORF2) indicated that the 4 pigs were infected with PCV2b. All 4 pigs were housed in one common room. Nasal swabs, oral swabs, fecal swabs, serum and fecal samples were collected weekly for Trial 1 until termination at 209 DPF.

2.1.2. Trial 2 (Experimentally PCV2-infected pigs)

The pigs for Trial 2 (n=5) originated from 3 litters which were all PCV2-naïve at the time of PCV2 inoculation since PCV2 DNA and anti-PCV2-antibodies were not detected in these pigs. All 5 pigs were housed in one common room. Nasal swabs, oral swabs, fecal swabs, serum and fecal samples were collected daily for the first two weeks and weekly thereafter. In addition, all pigs were injected with sterile saline intramuscularly at each sample collection time and the needle was subsequently flushed in the remaining sterile saline which was stored for analysis. Termination of Trial 2 was at 69 DPI.

2.1.3. Trial 3 (Bioassay)

The pigs for Trial 3 (n=28) originated from 6 litters and were PCV2-naïve (negative for PCV2 DNA and anti-PCV2-antibodies) at arrival. Serum samples were collected once a week. The pigs were randomly divided into 8 groups and rooms: Negative controls (n=3), Oral-IP [pooled oral samples via intraperitoneal (IP) administration; n=3], Fecal-IP (pooled fecal samples IP; n=3), Nasal-IP (pooled nasal samples IP; n=3), Oral-Oral (pooled oral samples via oral gavage; n=3), Fecal-Oral (pooled fecal samples via feed; n=4), Nasal-Nasal (pooled nasal samples IN; n=4), and Needle-IM (the same needle was used to inject an
experimentally-infected pig followed by injection of the naïve pigs; n=3). To avoid cross-contamination, four separate teams were responsible for inoculation and sampling procedures at each time point. Each team was responsible for two rooms and a change of clothes, masks, gloves, boots and a shower were required between rooms. Termination of Trial 3 was at 42 DPI.

2.2. Inoculation

2.2.1 Trial 2 (Experimentally PCV2-infected pigs)

At 21 days of age, each Trial 2 pig was inoculated with 5 ml of $10^{4.0}$ 50% tissue culture infectious dose (TCID<sub>50</sub>) per ml of a PCV2b isolate NC-16845 (Genbank #EU340258) produced from an infectious DNA clone as previously described (Fenaux et al., 2002). Three ml of inoculum was injected into the nares, and 2 ml was injected intramuscularly.

2.2.2. Trial 3 (Bioassay)

2.2.2.1. Inocula preparation

All samples used for inoculation (oral and nasal swabs, feces, needles) were obtained from Trial 2 pigs (experimentally PCV2-infected pigs) collected at 21 DPI.

For oral or nasal inocula, oral or nasal suspensions used for IP and IN injection (Oral-IP, Oral-Oral, Nasal-IP, and Nasal-Nasal groups) were prepared by vortexing the oral or nasal swabs for 15 sec, removing the swab and filtering the remaining solution first with a 0.45 µm and then with a 0.2 µm syringe filter (Fisher Scientific Inc., Pittsburgh, Pennsylvania, USA). The $\log_{10}$PCV2 genomic copies/ml in the inoculum was 7.4 for the Oral-IP and Oral-Oral groups, and 8.3 for the Nasal-IP and Nasal-Nasal groups.
For fecal inocula, pooled fecal material was used. For intraperitoneal injection (Fecal-IP group), a fecal suspension was prepared by mixing 2 g of feces with 12 ml of sterile saline solution. The mixture was vortexed for 2 min, centrifuged at 3,000 x g for 10 min at 10°C, and the supernatant was filtered by using 0.45 and 0.2 µm syringe filters (Fisher Scientific Inc.). For oral inoculation (Fecal-Oral group), 17.5 g pooled fecal material/pig was mixed with the normal feed ration of each pig. The log_{10} PCV2 genomic copies/ml was 8.3 for the Fecal-IP and Fecal-Oral groups.

For the needle preparation, an 18 GA 1.5 inch needle was used to inject 1 ml sterile saline into the muscle 5 cm distal to the base of the ear of a PCV2 viremic Trial 2 pig at 21 DPI. After usage, the needle was subsequently flushed three times in a sterile glass vial equipped with a plungeable rubber top containing 4 ml of sterile saline solution, aseptically removed from the syringe, placed into a sterile whirl pack bag, and transferred to a “clean” investigator and immediately used. PCV2 DNA was not detected in the sterile saline solution used to flush the needle.

2.2.2.2. Inoculation procedures

Inoculation was done at 42 days of age for all pigs in trial 3.

For intranasal inoculation, this was performed by using a 3 ml syringe to defuse 0.5 ml inoculum into each nostril for a total of 1 ml per pig.

For intraperitoneal inoculation, inoculation was performed by disinfecting the abdominal epithelium adjacent to the umbilical remnant with alcohol swabs (General Medical Corporation, Richmond, Virginia, USA) inserting a butterfly catheter (Jorgensen Laboratories, Inc., Loveland, Colorado, USA) into the peritoneal cavity, injecting 0.6 ml of the inoculum, and finally injecting 3 ml of sterile saline into the catheter.
For oral gavage, the inoculation procedure was performed by placing an 18 French red rubber catheter (Covidien, Mansfield, Massachusetts, USA) connected to a 12 ml syringe into the esophagus. For each pig, 2 ml of inoculum was placed into the syringe and flushed with 10 ml of water.

For oral inoculation via feeding, fecal material from Trial 2 pigs was mixed in with the normal feed ration, to ensure consumption of the material, the pigs were fed half of the normal amount of the ration the day prior to inoculation, the mixture was placed in separate areas of the pen for each pig, and the feeding was monitored by an observer.

For needle inoculation, the clean investigator affixed the needle to a new, sterile syringe, drew up 1 ml of sterile saline, which was then injected into the naïve animal in the muscle distal to the base of the ear.

2.3. Sample collection

One (Trial 1) or two (Trial 2) oral and nasal swabs were taken per animal using polyester swabs (Fisher Scientific Inc). Swabs were stored in 5 ml plastic tubes (Fisher Scientific Inc.) containing 1 ml of sterile saline solution (Fisher Scientific Inc.). Blood was collected in 8.5 ml serum separator tubes (Fisher Scientific Inc.), centrifuged at 2000 x g for 10 min at 4°C and serum was stored at -80°C until use. One fecal swab was taken per animal and stored in 5 ml plastic tubes containing 1 ml of sterile saline solution. In addition, in Trial 2 feces were collected from each animal and stored in individual 50 ml plastic centrifuge tubes (Fisher Scientific Inc.). At every sampling point, each pig was injected with 1 ml sterile saline solution to mimic vaccination procedure. The needle used to inject the pig was subsequently flushed three times in the vial containing 4 ml of sterile saline solution. Vials
containing the saline, feces, nasal secretions and oral secretions were stored at -80°C until testing.

2.4. Diagnostic testing

2.4.1. Detection of anti-PCV2-IgG antibodies

Serum samples were tested by an ORF2-based PCV2 IgG ELISA as previously described and were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater (Nawagitgul et al., 2002).

2.4.2. PCV2 DNA detection and quantification

DNA-extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, California, USA). DNA-extracts were used for quantification of the PCV2 genomic DNA copy numbers by a real-time PCR as previously described using ORF1-based primers (Opriessnig et al., 2003). The PCR reaction consisted of 25 µl PCR mixtures that contained 12.5 µl of commercially available master mix (TaqMan Universal PCR Master Mix, Applied Biosystems Inc., Foster City, California, USA), 2.5 µl DNA extract, 1 µl forward and reverse primers, 7.5 µl water, and 0.5 µl detection probe with concentrations of 10 µM. On each plate, five progressive 1:10 dilutions of a known copy number of PCV2 genomic DNA excised from a purified PCV2 DNA clone was included to generate a standard curve. Each plate was run in the sequence detection system (7500 Sequence Detection System; Applied Biosystems Inc.) under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Samples which did not generate a signal following 40 cycles were considered negative.

2.4.3. PCV2 sequencing
A nested PCR was used to amplify the entire ORF2 gene of PCV2 for sequencing as previously described (Opriessnig et al., 2006b). The PCR products were purified using the QIAquick PCR purification kit (Qiagen) per manufacturer’s instructions and sequenced at the Iowa State University DNA Sequencing facility. Sequences were analyzed with Sequence Scanner 1.0 (Applied Biosystems Inc.). Sequences were aligned using MegAlign (Lasergene DNASTar version 4.0.43, DNASTAR, Inc., Madison, Wisconsin, USA). PCV2 sequences were retrieved from the nucleotide sequence database of the National Center for Biotechnology Information (NCBI) to determine whether the viral sequences clustered with PCV2a or PCV2b. The ORF2 genes of the available sequences from GenBank database were aligned with three sequences obtained from the current study using the ClustalW method.

2.4.4. **PCV2 Immunohistochemistry (IHC)**

IHC detection of PCV2-specific antigen was performed on selected formalin-fixed and paraffin-embedded sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, spleen, Peyer’s patches, and thymus using a rabbit polyclonal antiserum (Sorden et al., 1999). PCV2-antigen scoring was done by a pathologist blinded to treatment groups. Scores ranged from 0 (no signal) to 3 (more than 50% of the lymphoid follicles contain cells with PCV2-antigen staining) (Opriessnig et al., 2004a).

2.4.5. **Assays to exclude the presence of other potential swine pathogens in the samples**

Serum samples collected at initiation and again at termination of Trials 1 (n=4), 2 (n=5) and 3 (n=8, one pig in each group) experiments were tested for the presence of specific antibodies to PRRSV with a commercial PRRSV ELISA (HerdChek® PRRS 2XR Antibody ELISA; IDEXX Laboratories, Inc. Westbrook, MA, USA), for the presence of PPV-specific antibodies by a hemagglutination inhibition (HI) assay (Mengeling et al.,
1988), and for the presence of SIV antibodies by an in house nucleoprotein NS1 ELISA (Opriessnig et al., 2010). Real-time PCRs for swine transmissible gastroenteritis virus (TGEV), PRRSV, *Mycoplasma hyopneumoniae* and SIV were performed according to the standard operating procedures of the Iowa State University Veterinary Diagnostic Laboratory. IHC staining for rotavirus antigen and *Lawsonia intracellularis* antigen was also performed according to the standard operating procedures of the Iowa State University Veterinary Diagnostic laboratory.

2.5. Necropsy

Animals were humanely euthanized with an overdose of pentobarbital (Vortech Pharmaceuticals, Dearborne, Michigan, USA) and necropsied on 209 DPF for Trial 1, 69 DPI for Trial 2, and 42 DPI for Trial 3. Macroscopic lung lesions, scored from 0 to 100% of the lung affected, and the size of lymph nodes, scored from 0 (normal) to 3 (enlarged four times the normal size), were estimated in a blinded fashion as described previously (Opriessnig et al., 2006a). Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), lung, tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

2.6. Histopathology

Microscopic lesions were evaluated by a pathologist (TO) blinded to treatment groups. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 4 (severe interstitial pneumonia) as described previously (Halbur
et al., 1995). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation, and scored from 0 (none) to 3 (severe).

Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 (normal) to 3 (severe) and for histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe) (Opriessnig et al., 2004a).

An overall microscopic lymphoid lesion score which accounts for lymphoid depletion, histiocytic inflammation, and PCV2-antigen present in lymphoid tissues was calculated for each pig as previously described (Opriessnig et al., 2004a) and the scores ranged from 0 (normal) to 9 (severe).

2.7. Statistics

The pig was considered the experimental unit for all analyses. Serological and viremia data were analyzed using a repeated measures analysis of variance (ANOVA) to test the null hypothesis that there was no effect of time on differences between samples (James and McCulloch, 1990). For the model, the sample type (fecal swab, oral swab, nasal swab, serum) was the fixed, independent variable and the continuous data (log$_{10}$ PCV2 genomic copies/ml or log$_{10}$ ELISA S/P ratios) were dependent variables. If a significant effect (P<0.05) was noted in the repeated measures ANOVA, a one-way ANOVA was performed at each DPF or DPI using the described model to assess significant differences between samples. For Trial 3 pigs, the time to seroconversion and viremia was calculated. These dependent variables were then used in a one-way ANOVA with the group as the independent variable. An effect was considered significant if P < 0.05. Statistical analysis was performed using JMP® (SAS Institute, Inc., Cary, North Carolina, USA).
RESULTS

3.1. Trial 1 (Naturally PCV2-infected pigs)

3.1.1. Clinical disease

A dry cough was noted in pig 1A on 121 DPF. Pig 1C developed bilateral rear leg ataxia on 146 DPF; however, the animal’s temperature was within normal limits (39.2°C) and the joints appeared normal. As pig 1C continued to have non-responsive bilateral rear-leg ataxia, pig 1C and pig 1D were moved to a separate room by 148 DPF to allow for better access to water and feed. After no response to treatment, pig 1C was humanely euthanized on 170 DPF. On 181 DPF, pig 1A developed slightly raised, red, multifocal-to-coalescing lesions on the ventral thorax, perineum and inner legs (rear and front) which resolved by the time of necropsy on 209 DPF.

3.1.2. Anti-PCV2-IgG antibodies

All four pigs were seropositive for IgG anti-PCV2 antibodies at 13 DPF (first sampling point) and remained seropositive throughout the study until 209 DPF without any significant decline (Fig. 1). In addition, all pigs were free of SIV-, PPV-, and PRRSV-specific antibodies at initiation and termination of the study.

3.1.3. PCV2 DNA quantification

All four pigs were PCV2 viremic at 13 DPF (first sampling point) and three animals were still viremic at 209 DPF. Interestingly, PCV2 DNA was consistently detected in serum samples until 126 DPF after which time PCV2 DNA was detected intermittently (Table 2). The detection of PCV2 DNA in fecal, nasal and oral swabs mirrored the PCV2 DNA
detection in the serum samples (Table 2). Mean group log_{10} PCV2 DNA genomic copies/ml for each sample type (fecal swab, nasal swab, oral swab and serum) are presented in Fig. 1. From 28 through 84 DPF, higher amounts of PCV2 DNA were detected in fecal and oral samples compared to that in nasal and serum samples, and the difference was significant (P<0.05) on 35, 49, 63, and 84 DPF.

3.1.4. Macropscopic lesions

Pig 1C had swollen rear hock filled with purulent material. Other gross lesions were not noted.

3.1.5. Microscopic lesions and PCV2 antigen detection by IHC

Microscopic lesions characteristic of PCVAD (lymphoid depletion and histiocytic replacement) were not present in pigs 1A, 1B, 1C or 1D. Low amounts of PCV2 antigen (positive staining detected in less than 10% of follicles) was detected by IHC in the tonsil and lymph node of two (1C and 1D) of the animals.

3.2. Trial 2 (Experimental PCV2-infection)

3.2.1. Clinical disease

Pig 2D developed dyspnea and diarrhea and had to be euthanized by 35 DPI. No clinical signs were noted in other animals.

3.2.2 Anti-PCV2-IgG antibodies

No anti-PCV2-IgG antibodies were detected in any of the animals prior to experimental inoculation (Fig. 2). Anti-PCV2-IgG antibodies were first detected in one animal at 11 DPI, one animal at 16 DPI, two animals at 19 DPI, and the remaining animals at 21 DPI. Anti-PCV2-IgG S/P ratios continued to increase in the majority of animals.
throughout the experiment (Fig. 2). Pig 2D which was euthanized on 35 DPI due to advanced clinical disease did not seroconvert to PCV2.

3.2.3. PCV2 DNA quantification

PCV2 DNA was not detectable in any pre-inoculation samples from any of the groups or in sera from negative control animals throughout the experiment. Mean group log$_{10}$ PCV2 DNA genomic copies/ml for the different sample types (feces, nasal swab, oral swab and serum) are presented in Fig. 2. All pigs became viremic by 1 DPI. All pigs showed a moderate decrease in the amount of PCV2 DNA detected from 2 to 4 DPI before resuming an increasing trend. Viremia peaked between 14 and 19 DPI with the exception of pig 2C which peaked at 42 DPI. The amount of PCV2 DNA present in fecal, nasal and oral swabs mirrored the amount detected in the sera. PCV2 DNA was detected in oral swabs, nasal swabs, fecal swabs and sera of the majority of the Trial 2 animals from 1 DPI until 7 DPI (Table 3). From 8 DPI until the termination of the study at 69 DPI, PCV2 DNA was detected in oral swabs, nasal swabs, fecal swabs and in sera from all animals. Peak virus shedding in oral, nasal and fecal samples appeared between 16 and 19 DPI. There were no significant differences in mean log$_{10}$ PCV2 genomic copies/ml among the sample types (fecal, nasal, oral, or serum) at 16 or 19 DPI. However, by 69 DPI, a significantly (P=0.04) higher amount of PCV2 DNA was detected in nasal swabs in comparison to fecal swabs and sera. PCV2 DNA was not detected in any of the saline samples used to flush the needles after IM injection.

3.2.4. No evidence of infection by other pathogens

Pig 2D (severe respiratory disease and diarrhea) was negative for the presence of TGEV antigen, rotavirus antigen or *Lawsonia intracellularis* antigen by IHC stains. The presence of PRRSV RNA, *Mycoplasma hyopneumoniae* DNA and SIV RNA was ruled out
by real-time PCR assays. Routine bacterial culture was negative. All pigs were free of SIV-, PPV-, and PRRSV-specific antibodies at initiation and termination of the study.

3.2.5. Microscopic lesions and PCV2 antigen detection by IHC

Microscopic lesions in pig 2D characterized by moderate, lymphoplasmacytic interstitial pneumonia with mild, histiocytic peribronchiolar and perivascular aggregates and type 2 pneumocyte hypertrophy and hyperplasia; mild, multifocal, suppurative alveolitis; severe lymphoid depletion and marked histiocytic replacement in germinal centers of multiple lymph nodes; severe, segmental, suppurative and histiocytic enterocolitis with numerous multinucleated giant cells; and moderate, multifocal, lymphoplasmacytic interstitial nephritis. Abundant amounts of PCV2 antigen were present within the cytoplasm of mononuclear cells in bronchiolar-associated lymphoid tissues, germinal centers and periarteriolar lymphoid sheaths of lymph nodes, and in the lamina propria and submucosa of the small intestine consistent with PCVAD.

Low amounts of PCV2 antigen were detected in the lymph node of one of the remaining four pigs (2B) at 69 DPI. In the same animal, moderate lymphoplasmacytic interstitial nephritis and mild, lymphoplasmacytic myocarditis was present. Mild lymphoplasmacytic interstitial nephritis was noted in one other animal.

3.3. Trial 3 (Bioassay)

3.3.1. Clinical disease

One animal in the Oral-Oral group was found dead at 8 DPI and had gross and microscopic lesions consistent with Mulberry Heart Disease (nutritional myocardiopathy).
No treatments or changes in diet were initiated for the remainder of the group. No other clinical disease was noted in the remaining animals.

3.3.2. Anti-PCV2-IgG antibodies

Anti-PCV2-IgG antibodies were not detected in any of the animals prior to experimental inoculation. The numbers of anti-PCV2 IgG positive animals in each of the experimental groups at each DPI and the mean time to seroconversion are presented in Table 4. There were no significant differences in mean time to seroconversion among groups. Seroconversion was noted in all three of the groups inoculated intraperitoneally, and in the groups orally inoculated with feces and nasal secretions. However, seroconversion was not detected in the group inoculated by oral gavage with oral secretions or in the group intramuscularly injected with contaminated needles. All pigs were free of SIV-, PPV-, and PRRSV-specific antibodies at termination of the study.

3.3.3. PCV2 DNA quantification

PCV2 DNA was detected in at least one animal from the Fecal-IP, Nasal-IP, Oral-IP, Nasal-Nasal, Fecal-Oral and Needle-IM groups (Table 5). Group mean log$_{10}$ PCV2 genomic copies/ml ± SE at 42 DPI and time to viremia (days ± SE) are shown in Table 5. No significant differences among groups were noted in time to viremia; however, there was a trend toward a shorter time to viremia in groups inoculated intraperitoneally.

3.3.4. PCV2 Sequencing and sequence analysis

The ORF2 gene of PCV2 recovered from one selected viremic pig in each group was sequenced and showed to have identical or near identical sequences (99-100% identity) when compared to that of the original inoculum.

3.3.5. Microscopic lesions and PCV2 antigen detection by IHC
Mean group overall microscopic lymphoid lesion scores and the number of IHC positive animals are present in Table 6. Microscopic lesions characteristic for PCV2 (lymphoid depletion and histiocytic replacement) were detected in lymphoid tissues from all groups except the negative control animals and the Oral-Oral group. There were no significant differences in overall microscopic lymphoid lesion score between groups. However, the most severe lesions were present in the Nasal-IP and Oral-IP groups and these groups also had the highest numbers of PCV2 IHC positive animals. Interestingly, animals in the Fecal-Oral group had a numerical trend toward more severe lesions and a higher number of PCV2 IHC positive animals compared to the animals in the Fecal-IP group.

3.3.6. No evidence of infection by other pathogens

Selected pigs in each group were free of SIV-, PPV-, and PRRSV-specific antibodies at initiation and termination of the study.

DISCUSSION

It is well documented that PCV2 infection is widespread in swine herds (Segalés et al., 2005a). The main objective of this study was to further investigate the shedding and infection dynamics of PCV2 in naturally and experimentally infected pigs to increase knowledge on which are the most important routes for horizontal transmission. PCV2 DNA was detected in oral, nasal and fecal swabs and in sera in naturally infected Trial 1 animals from 28 DPF (first sampling date in the research facility) up to 209 DPF (last sampling date) and in experimentally infected Trial 2 pigs from 1 DPI until 69 DPI (last sampling date). To our knowledge, 181 days (209 DPF minus 28 DPF) represents the longest documented period of PCV2 viremia under controlled conditions. Of studies which examined naturally-PCV2-
infected populations, PCV2 DNA was detected using PCR in animals at 25 weeks of age (Carasova et al., 2007), at 156 days of age (time of slaughter) (McIntosh et al., 2006), and in 25-50% of sampled 21-25 week old animals (Sibila et al., 2004). In experimental studies where the period of viremia is known, the longest documented period of PCV2 viremia by PCR is 140 DPI (study termination) (Opriessnig et al., 2010). In addition, successful isolation of PCV2 from spleen and distal ileum has been reported from pigs at 125 days post-infection (Bolin et al., 2001).

In Trial 1, peak PCV2 viremia (mean±SE of 5.32 ± 0.41 log_{10} genomic PCV2 copies per ml) and peak PCV2 shedding in oral, nasal, and fecal samples were observed at the beginning of the study (DPF 28) with a slow but gradual decline in the amount of PCV2 DNA in the different sample types analyzed thereafter. In contrast, peak PCV2 viremia was detected in Trial 2 pigs at 14 DPI with a mean±SE of 6.17±0.47 log_{10} genomic PCV2 copies per ml. Time of peak shedding in Trial 2 is similar to previous reports in which the highest number of PCR-positive animals was detected 14 to 21 DPI (Opriessnig et al., 2010). PCV2 peak shedding in oral, nasal and fecal samples in Trial 2 appeared between 16 and 19 DPI. During subsequent time points, the amounts of virus remained fairly stable in both trials with a downward trend in the remaining Trial 2 pen mates after removal of the PCVAD-affected pig (Pig 2D) at 35 DPI. This provides evidence to support the practice of removing or segregating sick animals from pen mates to reduce the viral load in the remaining animals in the same pen. Decreased viral load in the environment has been associated with decreased incidence of PCVAD (Larochelle et al., 2003).

Intermittent shedding was observed in both naturally-infected pigs (Trial 1) and pigs experimentally infected with PCV2 (Trial 2). In Trial 1 pigs, PCV2 DNA was consistently
shed in all sample types from 28 DPF until 97 DPF and was then there intermittently detected. Intermittent shedding was between 1 and 7 DPI and consistent thereafter for Trial 2 pigs. These discrepant results are likely due to the differences in exposure (experimental versus natural; vertical versus horizontal) and duration of testing. Experimental infection should result in a consistent time, route, and dosage of exposure. Assuming infected animals become resistant to future infections, the population would return to a negative status within a certain period of time due to lack of a susceptible population, and thus PCR detection would become sporadic as observed in Trial 1 starting on 97 DPF. In Trial 2, the duration of sampling was likely too short to see such an effect. In a similar study where experimentally infected animals were monitored until 140 DPI, PCV2 DNA was intermittently detected from 56 DPI to 130 DPI (Opriessnig et al., 2010). Early intermittent shedding of experimentally infected animals has also been documented. Following initial detection of PCV2 DNA at 1 DPI in experimentally infected pigs, the amount of PCV2 DNA decreased between 2 and 8 DPI before resuming an increasing trend at 9 DPI (Caprioli et al., 2006). In this study, samples were collected following oronasal inoculation of CD pigs and PCV2 DNA was detected in tonsillar and fecal swabs but not in sera which was attributed to the detection of PCV2 inoculum rather than true virus shedding (Caprioli et al., 2006). In contrast, pigs in Trial 2 were viremic at 1 DPI indicating that the DNA detected may be consistent with viremia. In Trial 1, animals with high concentrations of PCV2 DNA at 13 DPF were selected, indicating that PCV2 infection occurred prior to transfer of the animals to the research facility and therefore the expected early intermittent shedding pattern was not observed.

In the naturally infected Trial 1 pigs, clinical disease consistent with PCVAD was not observed in any of the animals. This is in contrast to Trial 2, where experimental inoculation
of PCV2 naïve pigs with PCV2 resulted in clinical signs consistent with PCVAD in 20% (1/5) of the animals. The reasons for this are unclear; however, passively-derived or active antibodies may have provided protection from clinical disease as previously reported (Allan et al., 2002). Alternatively, the initial infection dose may have been lower in the natural outbreak. Also, systemic infections occur in approximately 5 to 30% of infected populations (Opriessnig et al., 2007) and the differences between Trial 1 and trial 2 may be due to sample size. In addition, experimental reproduction of PCVAD is difficult and based on a meta-analysis of experimental studies, it is most successful when young (less than 3 weeks of age) CD pigs are infected with high doses of PCV2b and co-infected with a secondary pathogen (Tomas et al., 2008).

In Trial 2, seroconversion occurred between 11 and 21 DPI, and anti-PCV2 IgG antibodies remained elevated throughout the study. This is in agreement with several previous studies in which conventional SPF pigs were infected with PCV2 (Opriessnig et al., 2010; Segalés et al., 2005a). However, in Trial 1, the antibody response was unusual in that no decay of maternal antibodies was evident. Previous studies have shown that dam and piglet antibody titers are correlated, that pigs with lower S/P ratios have a higher and earlier likelihood of disease development, and that maternal antibodies decay between 4 to 12 weeks of age in PCV2 negative pigs (positive for anti-PCV2 IgG but negative for PCV2 DNA) (Opriessnig et al., 2004b) (Allan et al., 2002). However, the dynamics of the antibody response in PCV2-positive pigs (positive for both anti-PCV2 IgG and PCV2 DNA), as was the case for Trial 2 pigs, are largely unknown. Due to the consistent elevated levels of antibodies seen in the animals in Trial 1, there was likely a mixture of passively-acquired and active antibodies present in the piglets in addition to viable PCV2.
To our knowledge, the infectivity of PCV2 shedding in nasal secretions, fecal excretions and oral fluids or present on mechanical vectors such as needles has not been examined previously. Naïve Trial 3 animals were inoculated either by pooled nasal, oral, or fecal samples collected from experimentally infected Trial 2 pigs around the time of peak PCV2 shedding. Intraperitoneal inoculation with contaminated fecal, nasal and oral excretions resulted in PCV2 viremia and seroconversion in naïve pigs. Nasal secretions inoculated directly into the nares and fecal material fed to naïve animals were also infectious resulting in seroconversion of all four animals by 35 DPI. Feces fed to naïve animals resulted in transmission to two of four animals by 42 DPI. Additionally, viremia and microscopic lesions were noted in 1 of 3 pigs in the Needle-IM group, indicating that PCV2 may be spread among animals through vaccination procedures with a shared needle.

As all animals were housed together, it is possible that transmission within the room occurred once one animal became infected. Previous work has shown that the mean time for a newly infected animal to infect a susceptible animal is approximately 18 days when naïve animals are placed into the same pen as the experimentally-infected animals (Andraud et al., 2008). Therefore, it is possible that within the Fecal-Oral, Nasal-Nasal and Nasal-IP groups, not all the 3 pigs within each group were infected (primary infection) by the original inoculation, and that other pig(s) may subsequently become infected (secondary infection) from contact with the initial infected animal(s). While ideally each pig would have been housed individually to perform replicates of the experiment, the conditions of Trial 3 closely mimic field situations and thus answer the question of whether or not a group of animals can be infected by a particular route. Trends in the data suggest that the nasal route may be more effective than either the fecal or oral route. Additionally, at 69 DPI Trial 2 pigs had a
significantly (P<0.05) higher amount of PCV2 DNA in nasal swabs in comparison to fecal swabs and serum, suggesting that contact with nasal secretions may be a more important route for transmission than fecal-oral, especially in persistently infected animals.

CONCLUSION

This study documented the longest period of PCV2 viremia under controlled conditions (181 day observation period). It was also demonstrated that in both experimental and natural PCV2 infections, PCV2 is shed in high amounts in nasal, oral and fecal excretions for extended periods of time. Additionally, this study confirms that naïve animals can be infected with PCV2 present in nasal secretions, oral secretions and fecal material via the intraperitoneal route, with PCV2 present in feces via the oral route, with PCV2 present in nasal secretions via the intranasal route and potentially through contaminated needles.

CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Jeremy Johnson, Paul Thomas, Nicole Cressey and Rozann Stay for their help with animal work. The National Pork Board provided funding for this study; study sponsors had no involvement in study design, data collection, data analysis, data interpretation, writing of the manuscript or the decision to submit the manuscript for publication.
REFERENCES


Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. 40, 521-529.


Table 1. Experimental design. *Abbreviations used*: DPF=days post-farrowing; DPI=days post-inoculation; IM=intramuscular; IN=intranasal; IP=intraperitoneal.

<table>
<thead>
<tr>
<th>Trial</th>
<th>PCV2 status</th>
<th>Age at trial initiation</th>
<th>No. of rooms</th>
<th>Group designation</th>
<th>n</th>
<th>Inoculum</th>
<th>Route of infection</th>
<th>Time unit used</th>
<th>Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>Determined to be PCV2 PCR and ELISA positive at 13 DPF</td>
<td>28 days</td>
<td>1</td>
<td>Naturally infected pigs</td>
<td>4</td>
<td>None</td>
<td>Unknown</td>
<td>DPF</td>
<td>209 DPF</td>
</tr>
<tr>
<td>Trial 2</td>
<td>Naïve</td>
<td>21 days</td>
<td>1</td>
<td>Experimentally infected pigs</td>
<td>5</td>
<td>PCV2b</td>
<td>IM and IN</td>
<td>DPI</td>
<td>69 DPI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative controls</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oral-IP</td>
<td>3</td>
<td>PCV2b positive oral fluid</td>
<td>IP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fecal-IP</td>
<td>3</td>
<td>PCV2b positive feces</td>
<td>IP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nasal-IP</td>
<td>3</td>
<td>PCV2b positive nasal secretions</td>
<td>IP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oral-Oral</td>
<td>3</td>
<td>PCV2b positive oral fluid</td>
<td>Oral gavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fecal-Oral</td>
<td>4</td>
<td>PCV2b positive feces</td>
<td>Contaminated feed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nasal-Nasal</td>
<td>4</td>
<td>PCV2b positive nasal secretions</td>
<td>IN</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Needle-IM</td>
<td>3</td>
<td>Contaminated needle</td>
<td>IM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Detection of PCV2 DNA by PCR in oral, nasal, serum, or fecal samples (no. positive/total no. tested) of Trial 1 animals from days post farrowing (DPF) 97 to 209. Before DPF 97, all samples from all animals were positive.

<table>
<thead>
<tr>
<th>DPF</th>
<th>Oral swabs</th>
<th>Nasal swabs</th>
<th>Serum</th>
<th>Fecal swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>3/4</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>106</td>
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<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
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<tr>
<td>114</td>
<td>1/4</td>
<td>3/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>120</td>
<td>1/4</td>
<td>0/4</td>
<td>4/4</td>
<td>2/4</td>
</tr>
<tr>
<td>126</td>
<td>2/4</td>
<td>3/4</td>
<td>3/4</td>
<td>0/4</td>
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<td>4/4</td>
<td>2/4</td>
<td>2/4</td>
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<tr>
<td>141</td>
<td>3/4</td>
<td>3/4</td>
<td>2/4</td>
<td>3/4</td>
</tr>
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<td>147</td>
<td>1/4</td>
<td>2/4</td>
<td>4/4</td>
<td>2/4</td>
</tr>
<tr>
<td>154</td>
<td>1/4</td>
<td>3/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td>161</td>
<td>2/4</td>
<td>3/4</td>
<td>2/4</td>
<td>1/4</td>
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<tr>
<td>168</td>
<td>1/4</td>
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<td>1/4</td>
<td>1/4</td>
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<td>174</td>
<td>1/3</td>
<td>1/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>182</td>
<td>1/3</td>
<td>2/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>189</td>
<td>2/3</td>
<td>3/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>196</td>
<td>0/3</td>
<td>2/3</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>203</td>
<td>1/3</td>
<td>2/3</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td>209</td>
<td>0/3</td>
<td>1/3</td>
<td>3/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>
Table 3. Detection of PCV2 DNA by PCR in oral, nasal, serum, or fecal samples (no. positive/total no. tested) of Trial 2 animals from days post inoculation (DPI) 0 to 7. After DPI 7, all samples from all animals were positive until the termination of the study at DPI 69.

<table>
<thead>
<tr>
<th>DPI</th>
<th>Oral swabs</th>
<th>Nasal swabs</th>
<th>Serum</th>
<th>Fecal swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>1</td>
<td>4/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
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<tr>
<td>2</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
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<tr>
<td>3</td>
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<td>5/5</td>
<td>5/5</td>
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<tr>
<td>4</td>
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<td>5/5</td>
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<td>6</td>
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<td>5/5</td>
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<tr>
<td>7</td>
<td>3/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>
Table 4. Serological response in Trial 3 animals: Number of anti-PCV2 IgG positive animals/total number of animals following intramuscular inoculation with a contaminated needle (Needle-IM); intraperitoneal inoculation with feces, nasal secretions or oral secretions (Fecal-IP, Nasal-IP, Oral-IP respectively); oral gavage with oral secretions (Oral-Oral); or oral inoculation with pooled fecal material (Fecal-Oral). Serum samples were considered positive if the sample-to-positive (S/P) ratio was greater than 0.2.

<table>
<thead>
<tr>
<th>Group</th>
<th>DPI</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>42</th>
<th>Time to Seroconversion Mean days ± SE</th>
<th>DPI 42 Anti-PCV2-IgG S/P ratio Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral-IP</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
<td></td>
<td>35.0 ± 4.0</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>Fecal-IP</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td></td>
<td>25.7 ± 2.3</td>
<td>0.67 ± 0.05</td>
</tr>
<tr>
<td>Nasal-IP</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td></td>
<td>30.3 ± 4.7</td>
<td>0.54 ± 0.15</td>
</tr>
<tr>
<td>Oral-Oral*</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td></td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>Fecal-Oral</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
<td>1/4</td>
<td>2/4</td>
<td></td>
<td>35.0 ± 7.0</td>
<td>0.22 ± 0.16</td>
</tr>
<tr>
<td>Nasal-Nasal</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
<td>2/4</td>
<td>4/4</td>
<td>4/4</td>
<td></td>
<td>29.8 ± 3.4</td>
<td>0.64 ± 0.12</td>
</tr>
<tr>
<td>Needle-IM</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td></td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*One animal in this group died of Mulberry Heart Disease on DPI 8.  
**Not applicable.
Table 5. PCV2 viremia in Trial 3 animals: Number of of PCV2 viremic animals/total number of animals following intramuscular inoculation with a contaminated needle (Needle-IM); intraperitoneal inoculation with feces, nasal secretions or oral secretions (Fecal-IP, Nasal-IP, Oral-IP respectively); oral gavage with oral secretions (Oral-Oral); or oral inoculation with pooled fecal material (Fecal-Oral). Time to viremia (days ± std. err) and group mean log_{10} genomic copies/ml ± std. err of PCV2 at days post inoculation (DPI) 42 are provided.

<table>
<thead>
<tr>
<th>Group</th>
<th>DPI</th>
<th>Time to viremia</th>
<th>DPI 42 Log_{10} PCV2 genomic copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Oral-IP</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Fecal-IP</td>
<td>0/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Nasal-IP</td>
<td>0/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Oral-Oral*</td>
<td>0/4</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Feces-Oral</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Nasal-Nasal</td>
<td>0/4</td>
<td>2/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Needle-IM</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*One animal in this group died of Mulberry Heart Disease at DPI 8.
**Not applicable.
Table 6. Microscopic lesions (Group mean ± SE) and number of PCV2 antigen (as determined by immunohistochemistry) positive Trial 3 pigs /total number of Trial 3 pigs per group following intraperitoneal inoculation with feces, nasal secretions or oral secretions (Fecal-IP, Nasal-IP, Oral-IP respectively); oral gavage with oral secretions (Oral-Oral); oral inoculation with pooled fecal material (Fecal-Oral); intramuscular inoculation with a contaminated needle (Needle-IM). One group (Negative) remained un-inoculated.

<table>
<thead>
<tr>
<th>Group</th>
<th>Overall lymphoid lesion score*</th>
<th>PCV2 antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral-IP</td>
<td>3.7±0.6</td>
<td>3/3</td>
</tr>
<tr>
<td>Fecal-IP</td>
<td>0.7±0.7</td>
<td>1/3</td>
</tr>
<tr>
<td>Nasal-IP</td>
<td>3.7±1.4</td>
<td>3/3</td>
</tr>
<tr>
<td>Oral-Oral</td>
<td>0.0±0.0</td>
<td>0/3</td>
</tr>
<tr>
<td>Fecal-Oral</td>
<td>1.4±0.5</td>
<td>3/4</td>
</tr>
<tr>
<td>Nasal-Nasal</td>
<td>2.1±1.2</td>
<td>2/4</td>
</tr>
<tr>
<td>Needle-IM</td>
<td>0.8±0.8</td>
<td>1/4</td>
</tr>
<tr>
<td>Negative</td>
<td>0.0±0.0</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* An overall microscopic lymphoid lesions score which accounts for lymphoid depletion, histiocytic inflammation, and PCV2-antigen present in lymphoid tissues was calculated for each pig and ranged from 0=normal to 9=severe.
**Figure 1.** Mean group log$_{10}$ genomic PCV2 copies/ml oral fluid, nasal secretions, serum and feces (lines) and mean group ELISA sample-to-positive (S/P) ratio (bars) in four pigs naturally infected with PCV2 (Trial 1). An S/P ratio of 0.2 or greater was considered to be positive.

An S/P ratio of 0.2 or greater was considered to be positive.
Figure 2. Mean group log_{10} genomic PCV2 copies/ml oral fluid, nasal secretions, serum and feces (lines) and mean group ELISA sample-to-positive (S/P) ratio (bars) in five pigs experimentally infected with PCV2 (Trial 2). An S/P ratio of 0.2 or greater was considered to be positive.
CHAPTER 5. Efficacy of experimentally-produced spray-dried plasma on infectivity of porcine circovirus type 2 (PCV2)

A paper submitted to the Journal of Animal Science

Abby R. Patterson, Darin M. Madson, Tanja Opriessnig

ABSTRACT: The value of incorporating spray-dried plasma (SDP) into the diet of weanling pigs to improve feed intake and growth performance has been well documented. However, limited work has been done to confirm that the spray-drying process eliminates all viral contaminates including porcine circovirus type 2 (PCV2). To determine the effect of spray-drying on PCV2 infectivity, colostrum-fed, crossbred, specific-pathogen-free (SPF) pigs were inoculated with PCV2-contaminated SDP intraperitoneally (SDP-IP) or by oral gavage (SDP-OG), inoculated intraperitoneally with PCV2-positive plasma (POS), or left uninoculated (NEG). The plasma used for the experimentally produced SDP was collected from a SPF pig experimentally-infected with a PCV2b isolate. Pigs in the NEG group remained seronegative and PCV2 viremia was not detected. All pigs in the POS group became PCV2 viremic by 14 days post-inoculation (DPI) and seroconverted by 28 DPI. In the SDP-IP group, all pigs became viremic by 35 DPI and seroconverted by 49 DPI. In the SDP-OG group, all animals became viremic by 35 DPI and 2/3 pigs seroconverted by 35 DPI. There were no significant differences between anti-PCV2-IgG antibody sample-to-positive (S/P) ratios among pigs in the POS, SDP-OG, or SDP-IP groups. This work provides direct evidence that the experimental spray-drying process used in this study was not effective in
inactivating PCV2b in the plasma of a PCV2-infected pig based on a swine bioassay using PCV2 naïve pigs. This work suggests that spray dried plasma sourced from pigs could represent a biosecurity risk for the industry.

**Key words:** porcine circovirus type 2, seroconversion, spray-dried plasma, viremia

**INTRODUCTION**

Porcine circovirus type 2 (PCV2) is the primary component of PCV associated disease (PCVAD), a systemic infection that can result in mortality rates of 70-80% in clinically affected pigs (Opriessnig et al., 2007). While the virus was first associated with disease in the late 1990’s (Allan et al., 1998), between 2004 and 2005 an increase in the number and severity of PCVAD cases was reported in Canada (Carman et al., 2006), and in Kansas, Iowa and North Carolina (Cheung et al., 2007). Interestingly PCV2b, a strain of PCV2 not previously isolated in North America, was identified (Cheung et al., 2007). The source and route of transmission of PCV2b in North America is currently unknown. Potential routes of PCV2 transmission include direct contact with infected animals and contaminated fomites or vertical transmission. Horizontal transmission of PCV2 through spray-dried plasma (SDP) products is another hypothesis.

The value of incorporating SDP protein into the diet of weanling pigs to improve feed intake and growth performance, especially following disease challenge has been well documented (Coffey and Cromwell, 2001; Hansen et al., 1993; Lawrence et al., 2004; Torrallardona, 2010; Zhao et al., 2007). PCV2 is a small circular DNA virus and has been shown to be extremely resistant to inactivation as evidenced by a 1-log reduction after dry-
heat treatment of freeze-dried PCV2 at 120°C for 30 min (Welch et al., 2006) and resistance to a 15 min 70°C heat treatment in cell culture (O'Dea et al., 2008).

Commercially manufactured SDP protein containing $2.47 \times 10^5$ PCV2 genomic copies per ml fed to weaning pigs did not result in seroconversion or viremia during the 45 day observation period (Pujols et al., 2008). However, pigs had low levels of maternally-derived PCV2 antibodies before initiation of the trial based on an immunoperoxidase monolayer assay (IPMA) (Pujols et al., 2008).

To investigate whether PCV2 remains infectious after the spray-drying process, SDP derived from a pig experimentally-infected with PCV2b was tested in a swine bioassay using PCV2 naïve animals.

**MATERIALS AND METHODS**

*Animals and housing*

The experimental protocol in this study was approved by the Iowa State University Institutional Animal Care and Use Committee. Colostrum-fed, crossbred, specific-pathogen-free (SPF) pigs were purchased from a herd that is routinely tested for major swine pathogens and known to be free of PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV) and swine influenza virus (SIV). Twelve pigs obtained from 2 litters were weaned at 3 weeks of age and transported to the Livestock Infectious Disease Isolation Facility at Iowa State University, Ames, Iowa. Upon arrival, the pigs were ear-tagged, randomly divided into groups of three pigs using a random permutation generator (freeware available online at http://www.randomization.com) and housed in four separate rooms; there were no partitions separating the three animals within each room. Each room
had 18 m² of solid concrete floor space, separate ventilation systems and one nipple drinker. All groups were fed a balanced, pelleted, complete feed ration free of animal proteins (with the exception of whey) and antibiotics (Nature’s Made, Heartland Coop, Alleman, Iowa, USA) once a day.

**Experimental design**

Pigs were left un-inoculated (NEG), inoculated with an experimentally-produced reconstituted PCV2-infected SDP product intraperitoneally (SDP-IP) or by oral gavage (SDP-OG), or inoculated intraperitoneally with plasma from a PCV2-infected pig (POS). Blood samples were collected weekly after inoculation for 7 weeks and tested for the presence of anti-PCV2-IgG antibodies and PCV2 DNA.

**PCV2-contaminated plasma source**

The source of plasma inoculum was blood from a colostrum-fed, crossbred, SPF pig experimentally-infected with $5 \times 10^{4.0}$ 50% tissue culture infectious dose (TCID₅₀) of PCV2b (GenBank # EU340258) at 3 weeks of age as part of a separate study (A. Patterson, unpublished data). At 35 days post inoculation (DPI), the pig was euthanized with an overdose of pentobarbital (Fatal Plus®, Vortech Pharmaceutical, Dearborn, Michigan, USA) due to development of clinical signs consistent with PCVAD including severe dyspnea, diarrhea, and loss of condition. After euthanasia, 2 liters of blood were collected in jars containing 14,300 USP Heparin Units (Hospira, Inc., Lake Forest, Illinois, USA) per liter of blood. The plasma was immediately centrifuged at 2000 $\times$ g for 10 min at 4°C in 50 ml centrifuge tubes and stored at 4°C until use. The plasma was tested for the presence of PCV2 DNA by quantitative PCV2 PCR; 7.75 log₁₀ PCV2 genomic copies per ml of plasma were detected. The diagnosis of PCVAD in the donor pig was further confirmed by the presence of
intense PCV2 antigen staining by immunohistochemistry (IHC) (Sorden et al., 1999) in the lungs, intestine and lymphoid tissues. Transmissible gastroenteritis virus (TGEV), rotavirus, and *Lawsonia intracellularis* were not detected by IHC. PCR tests for PRRSV (Prickett et al., 2007), SIV (Richt et al., 2004), and *Mycoplasma hyopneumoniae* (Calsamiglia et al., 1999) were negative. The IHC assays and PCR tests were performed according to the standard operating procedures for the Iowa State University Veterinary Diagnostic Laboratory.

**Spray-drying process**

Three-hundred ml of the collected plasma was spray-dried using a bench-top spray dryer (Yamato Model ADL310, Yamato Scientific Co., LTD, Tokyo, Japan). To ensure that the spray dryer was not contaminated with PCV2 before the initiation of the run, it was disinfected with an oxidizing disinfectant (Virkon® S, Dupont, Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut, USA) according to the manufacturers’ recommendations. Ten swabs were then taken from various components of the apparatus, placed in sterile saline and were confirmed to be PCV2 negative by real-time PCR (Opriessnig et al., 2003).

For drying of the plasma, the bench-top spray-drier manufacturer’s recommendations were followed. A 0.4 mm nozzle was used with the following parameters: $T_{\text{inlet}}$ (inlet air temperature) of 166°C, aspiration rate of 0.6 m$^3$/min, $T_{\text{outlet}}$ (outlet temperature) of 67°C, and an 820 ml/hr sample flow rate under 0.1 MPa of pressure. The resulting SDP product was stored at 4°C until use at which time it was reconstituted in sterile saline to a concentration of 0.33 g/ml. This concentration was based on the amount of available product, the number of animals in the study and the amount of saline needed to reconstitute the SDP product. Some of the differences between the experimentally-produced SDP used in this study and
commercially-produced SDP include, but are not limited to, the source animal(s), disease status, pooling effects, processing temperatures, product retention time and post drying conditions (Table 1).

**Inoculation**

Pigs in the SDP-IP and SDP-OG groups were inoculated with 3 ml of the reconstituted SDP product as described above. Pigs in the POS group were inoculated intraperitoneally with 3 ml of the untreated plasma. Pigs in the NEG group were sham-inoculated intraperitoneally with 3 ml of sterile saline.

**Anti-PCV2-IgG antibodies**

Blood samples were collected on the day of inoculation, and weekly thereafter until 49 DPI. The blood was collected in 8.5 ml serum separator tubes (Fisher Scientific, Pittsburgh, Pennsylvania, USA), immediately centrifuged at 2000 \( x \) g for 10 minutes at 4°C and stored at -80°C until use. Serum samples were tested by an open-reading frame (ORF) 2-based PCV2 IgG ELISA as previously described and were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater (Nawagitgul et al., 2002).

**PCV2 DNA quantification**

DNA-extraction on all serum samples was performed using the QIAamp® DNA Mini Kit (Qiagen, Valencia, California, USA). DNA-extracts were used for quantification of the PCV2 genomic DNA copy numbers by real-time PCR. Previously described primers for ORF1 of PCV2 (Opriessnig et al., 2003) were used for quantification of the PCV2 genomic DNA copy numbers by real-time PCR. The PCR reaction consisted of 25 µl PCR mixtures that contained 12.5 µl of commercially available master mix (TaqMan Universal PCR Master Mix, Applied Biosystems), 2.5 µl DNA extract, 1 µl forward and reverse primers, and 0.5 µl
detection probe with concentrations of 10 µM. On each plate five progressive 1:10 dilutions of a known copy number of PCV2 genomic DNA excised from a purified PCV2 DNA clone were included to generate a standard curve. Each plate was run in the sequence detection system (7500 Sequence Detection System, Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

**Sequencing**

PCR products amplified from virus recovered from a 49 DPI serum sample from one animal in the SDP-IP, SDP-OG and POS groups was sequenced and compared with the inoculum. A nested PCR was used to amplify the entire ORF2 gene for sequencing (Opriessnig et al., 2006b). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, California, USA) per the manufactures directions and sequenced at Iowa State University DNA facility. Sequences were analyzed with Sequence Scanner 1.0 (Applied Biosystems) and compared with the sequence of the inoculum using the BLAST (basic local alignment search tool) (Zhang et al., 2000).

**Necropsy**

All pigs were necropsied at 49 DPI. Severity of macroscopic lung lesions (scored from 0 to 100% of the lung affected) and the size of lymph nodes (score ranged from 0 to 3; 0 [normal], 1 [two times the normal size], 2 [three times the normal size], 3 [four times the normal size]) were estimated in a blinded fashion as described previously (Opriessnig et al., 2006a). Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological
examination.

**Histopathology and immunohistochemistry (IHC)**

Microscopic lesions were evaluated by a pathologist blinded to treatment groups. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 4 (severe interstitial pneumonia) as described previously (Halbur et al., 1995). Sections of heart, liver, kidney, ileum and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 (normal) to 3 (severe) and histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe) (Opriessnig et al., 2004).

Detection of PCV2-specific antigen in selected formalin-fixed and paraffin-embedded sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, spleen, Peyer’s patches, and thymus was performed by using IHC and a rabbit polyclonal antiserum as described previously (Sorden et al., 1999). PCV2-antigen scoring was done by a pathologist blinded to treatment groups. Scores ranged from 0=no signal to 3=more than 50% of the lymphoid follicles contain cells with PCV2-antigen staining (Opriessnig et al., 2004).

An overall microscopic lymphoid lesions score which accounts for lymphoid depletion, histiocytic inflammation, and PCV2-antigen present in lymphoid tissues was calculated for each pig as previously described (Opriessnig et al., 2004) and ranged from 0=normal to 9=severe.

**Statistical analysis**

To test the null hypothesis that there was no effect of time on differences between the
groups, a repeated measures analysis of variance (ANOVA) was used (James and McCulloch, 1990). The experimental unit was the individual pig. For the model, group (NEG, SDP-IP, SDP-OG, and POS) was the fixed, independent variable and the continuous data (log transformed genomic copies/ml or anti-PCV2-IgG S/P ratios) were dependent variables. If a significant effect ($P < 0.05$) was noted in the repeated measures analysis, a one-way ANOVA was performed at each DPI using the previously described model to assess significant differences between groups. For statistical analysis of lymphoid lesions, a one-way ANOVA was performed using the overall microscopic lymphoid lesions score as a dependent variable and group (NEG, SDP-IP, SDP-OG, and POS) as the fixed, independent variable in the ANOVA. If an ANOVA was significant, pairwise comparisons using the Tukey-Kramer adjustment were done to determine which groups were different. Statistical analysis was performed using JMP® 7 (SAS Institute, Inc., Cary, North Carolina, USA).

RESULTS

Clinical presentation and macroscopic lesions

Clinical disease was not observed in any of the pigs for the duration of the study. No remarkable gross lesions were observed. Individual pigs in the POS, SDP-IP and SDP-OG groups had slightly enlarged mediastinal lymph nodes compared to the NEG group.

Anti-PCV2-IgG antibody levels

Group mean and standard errors for anti-PCV2-IgG antibody S/P ratios are presented in Fig. 1. Pigs within the NEG group remained seronegative throughout the duration of the trial. By DPI 21, 2/3 pigs in the POS group seroconverted; by DPI 28 all three pigs had seroconverted. In the SDP-IP group, 2/3 pigs seroconverted on DPI 35; by DPI 49 all pigs
had seroconverted. In the SDP-OG group, 2/3 pigs seroconverted by DPI 35 and one animal remained negative through DPI 49. Throughout the trial, there were no significant differences between anti-PCV2-IgG antibody S/P ratios among pigs in the POS, SDP-OG, or SDP-IP groups. At 49 DPI, POS, SDP-OG and SDP-IG pigs had significantly higher group mean S/P ratios compared to the NEG group.

**PCV2 DNA quantification and sequencing**

Group means and standard errors for log$_{10}$ transformed PCV2 genomic copies/ml are presented in Fig. 2. Pigs within the NEG group remained negative throughout the duration of the trial. In the POS group, all pigs were viremic by DPI 14. In the SDP-IP group, 1/3 pigs were viremic on DPI 14, 2/3 pigs were viremic on DPI 21, and by DPI 35 all three pigs were viremic. In the SDP-OG groups, one pig became viremic at DPI 14, 2/3 pigs were viremic by DPI 28 and all three pigs were viremic at DPI 35. A difference ($P < 0.05$) in amount of PCV2 DNA was observed between the POS and the SDP-IP at DPI 14 and between the SDP-IP and SDP-OG groups at DPI 49 (Fig. 2). No other differences were noted between the POS, SDP-IP and SDP-OG groups. By DPI 35, all groups had significantly higher amounts of PCV2 DNA compared to the NEG group. Sequence analysis of the PCV2 recovered from one pig from each group which became viremic had 100% similarity with the inoculum.

**Histopathology and immunohistochemistry**

Pigs within the NEG control group had no microscopic lesions associated with PCVAD (overall microscopic lymphoid lesions score of 0) and no lung lesions. Pigs within the POS control group had a mean overall microscopic lymphoid lesions score ($\pm$ standard error) of 3.3 ± 0.2. Specifically, there was mild-to-moderate lymphoid depletion and histiocytic replacement of lymphoid follicles in 3 of 3 pigs which was associated with low
levels of PCV2 antigen in 2 of 3 pigs in the POS control group. Pigs within the SDP-IP and SDP-OG groups had a mean overall microscopic lymphoid lesions score of 3.7±2.8 and 2.7±1.2, respectively. Specifically, there was moderate lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues in 1 of 3 animals and low-to-moderate amounts of PCV2 antigen were demonstrated in 3 of 3 animals in the SDP-IP group. In the SDP-OG group, there was mild lymphoid depletion in lymphoid tissues in 3 of 3 animals and low-to-moderate amounts of PCV2 antigen were present 2 of 3 animals. There was no significant difference in overall microscopic lymphoid lesions scores among the POS, SDP-IP and SDP-OG groups. Lesion scores in all groups were numerically higher in comparison to NEG pigs; however, the difference was significant only for the SDP-IP group. Significant differences in lung lesions were not observed among treatment groups.

**DISCUSSION**

The source of the apparent introduction of PCV2b into multiple locations in North America during a short period of time in 2004 is currently unknown. Before 2004, only the PCV2a genotype had been isolated (Larochelle et al., 2002). Between 2004 and 2005 a marked increase in the numbers of severe PCVAD cases were noted in Canada (Carman et al., 2006) and in the United States (Cheung et al., 2007). Sequence analysis of these cases confirmed the first occurrence of PCV2b in North America.

PCV2 is known to be shed in feces, urine, nasal and oral secretions (Segalés et al., 2005) and remain in tissues for extended periods of time (Bolin et al., 2001). Vertical transmission of the virus has also been documented (Madson et al., 2009). The combination of multiple shedding routes with the viruses’ ability to withstand environmental stress, leads
to the possibility of horizontal transmission. Potential routes for horizontal transmission include direct contact with infected animals or indirect contact with contaminated fomites, aerosols, or mechanical vectors. Based on previous work, direct contact with infected animals is likely more efficient than other sources of transmission (Andraud et al., 2008).

One potential mechanism for the spread of PCV2b that has not been thoroughly investigated is transmission through SDP products. However, several studies have reported lack of viability of viruses in SDP. Pseudorabies virus (PRV) and PRRSV were not detected in a study where bovine plasma was spiked with the aforementioned viruses, spray-dried and tested in cell culture for presence of infectious virus (Polo et al., 2005). In addition, commercially produced SDP product incorporated into the diet of weanling pigs did not result in seroconversion to any of the tested viruses (Polo et al., 2005). Similarly, when porcine plasma was spiked with $10^6$ TCID$_{50}$ swine vesicular disease virus (SVDV) per ml, virus was not detected in spray-dried samples by virus isolation (Pujols et al., 2007). In contrast to PRRSV, PRV and even other stable, non-enveloped viruses such as SVDV (Turner and Williams, 1999), PCV2 has been shown to be extremely resistant and to maintain viability at temperatures of 60°C for 24 hrs and 75°C for 15 min (O'Dea et al., 2008; Welch et al., 2006).

The bench-top model of a spray-dryer used in this experiment uses a co-current flow of heated air and atomized spray in an open-cycle system to evaporate moisture from an aqueous or organic solution (Buchi training papers - spray drying; Buchi Labortechnik AG, 1997-2002). Although inlet temperatures of 240°C can be achieved during this process, the time in which the product is exposed to this temperature is short. This design enables drying of plasma products without the destruction of various proteins including antibodies to PCV2,
Mycoplasma sp., PRRSV, TGEV and SIV (Borg et al., 2002). There are differences between a bench-top model and commercial units, the main one being size which affects the retention time of the product within the chamber. The retention time is balanced through control of various parameters (inlet temperature, liquid feed rate, etc.) to minimize damage to the proteins and maximize the efficiency of the drying process (Maa et al., 1998).

It has been demonstrated that when SDP products are incorporated into the diets of weanling pigs, dietary plasma proteins promote immune modulation of pro- and anti-inflammatory cytokines resulting in improvements in growth parameters (Moreto and Perez-Bosque, 2009). This same process which allows proteins to remain stable, may allow extremely resistant viruses such as PCV2 to retain infectivity. For example, the outlet temperature can be generalized as the maximum product temperature (Buchi training papers - spray drying; Buchi Labortechnik AG, 1997-2002). Using this generalization, the temperature of the experimentally-produced SDP in this experiment was between 67 to 71°C; similar to previously reported temperatures where PCV2 remained viable for 15 min (75°C) or 24 hrs (60°C) (O'Dea et al., 2008; Welch et al., 2006). Therefore, the ability of PCV2 to cause seroconversion and viremia in naïve animals in this study was not unexpected. The parameters used in this study were based on recommendations of the manufacturer of the bench-top spray-dry unit used. Further determination of the necessary inactivation temperature for PCV2 by this method is warranted.

Based on the development of viremia and time of seroconversion, it is highly probable that within the SDP-IP and SDP-OG group intra-pen transmission occurred. Previous work has shown that the mean time for a newly infected animal to infect a susceptible animal is approximately 18 days when naïve animals are placed into the same pen
as experimentally infected animals (Andraud et al., 2008). While ideally each pig would have been housed individually to perform replicates of the experiment, the conditions of the trial answer the question of whether a group of animals can be infected with PCV2 through exposure to an experimentally generated SDP product containing PCV2. However, this study does not indicate the relative magnitude and importance of this source of infection under field conditions.

Transmission of PCV2 from external sources into the experimental rooms was controlled in this study by utilizing multiple sampling teams whenever possible. If entering multiple rooms on the same day was absolutely necessary, a shower was taken between rooms and different face masks, gloves and coveralls were worn in each room. Throughout the study, negative control animals housed under the same conditions as experimental groups failed to seroconvert or become viremic. Additionally, sequence analysis of the PCV2 recovered from one viremic pig from each group confirmed 100% similarity with the inoculum.

In contrast to the results of this experiment, in a study in which SDP was incorporated into the diet of three-to-four week old SPF pigs, neither seroconversion nor viremia was detected (Pujols et al., 2008). In contrast to the present study, in which PCV2-naïve pigs were utilized, pigs with low levels of passively-derived anti-PCV2-IgG as determined by IPMA (all pigs were negative based on a capture ELISA) were used by Pujols et al. (2008) which is more representative of commercial pigs. The increased sensitivity of the IPMA in comparison to the capture ELISA, likely indicates that the levels of passively-acquired antibody were low (Pujols et al., 2008). Possible passively-acquired antibodies that may have remained undetected by the ORF-2 ELISA in the animals used in the current study can be
ruled out as the PCV2-naïve status of the source herd was monitored over time and also confirmed by additional serological assays on the entire sow population (data not shown). Previously, in animals with high levels of passively-acquired antibodies decreased viremia and seroconversion following experimental challenge with PCV2 was observed; however, low levels of passively acquired antibody titers were not found to be generally protective (McKeown et al., 2005). As anti-PCV2 antibodies can mask or delay infection we decided to use a bioassay model based on naïve pigs.

Additional differences to the study by Pujols et al. (2008) include the use of different sources of SDP and a different route of inoculation. In the current study, plasma containing $5.63 \times 10^7$ PCV2 genomic copies per ml from an experimentally inoculated pig with clinical and histological evidence of PCVAD was used to directly inoculate the animals in the POS control group and to generate the SDP product used to inoculate the animals in the SDP-IP and SDP-OG groups. In the study by Pujols et al. (2008), commercially-processed SDP containing $2.47 \times 10^5$ PCV2 DNA copies/ml was used. While information is not available on the minimum infectious dose for PCV2, in previous work by the authors, oral administration of meat containing $10^4$ PCV2 DNA copies per ml of homogenized tissue resulted in seroconversion and viremia in naïve animals (Opriessnig et al., 2009). In published experimental studies, the most common route of infection is the intra- or oronasal route using doses ranging from $10^2$ TCID$_{50}$ per ml to $10^6$ TCID$_{50}$ per ml (Tomás et al., 2008). In the current study, the main objective was to determine if SDP was infectious. Therefore, pigs were inoculated via intraperitoneal injection and by oral gavage in a tightly controlled bioassay model. In contrast, Pujols et al. (2008) incorporated the SDP in the diet of pigs for 45 days which more closely simulates field conditions.
This work provides direct evidence that experimentally-produced spray-dried porcine plasma collected from a PCV2b-experimentally infected pig is infectious to naïve animals through the intraperitoneal and oral gavage routes. However, commercially produced products utilize pooled plasma from clinically healthy pigs and it is not appropriate to fully extrapolate results from this experimentally-produced SDP process to the commercially-produced spray dried porcine plasma process and product used in the swine industry today.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Jeremy Johnson and Paul Thomas for their help with animal work and the National Pork Board for funding the study.

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**Table 1.** Summary of important differences in the production of the experimentally produced spray-dried plasma used in this study and typical products produced for commercial use.

<table>
<thead>
<tr>
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<th><strong>Experimental plasma used</strong></th>
<th><strong>Typical commercial plasma</strong></th>
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<tbody>
<tr>
<td><strong>Overall health status</strong></td>
<td>Clinical signs of PCVAD (severe dyspnea, diarrhea, and loss of condition) requiring euthanasia</td>
<td>Ambulatory and free of clinical signs of disease; inspected by federal authorities and passed as fit for slaughter for human consumption</td>
</tr>
<tr>
<td><strong>Age at plasma collection</strong></td>
<td>8 weeks</td>
<td>25 weeks (mature; market weight)</td>
</tr>
<tr>
<td><strong>PCV2 status</strong></td>
<td>High PCV2 viremia and no anti-PCV2 IgG antibodies</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Pooling</strong></td>
<td>Not pooled (1 pig)</td>
<td>Pooled (8,000 to 20,000 pigs)</td>
</tr>
<tr>
<td><strong>Inlet temperature</strong></td>
<td>166°C</td>
<td>200°C to 240°C</td>
</tr>
<tr>
<td><strong>Outlet temperature</strong></td>
<td>67°C to 71°C</td>
<td>80°C to 90°C</td>
</tr>
<tr>
<td><strong>Dwell/Retention time (drying chamber)</strong></td>
<td>Less than 1 sec</td>
<td>20 sec to 1 min</td>
</tr>
<tr>
<td><strong>Post drying configuration (cyclone, bag house)</strong></td>
<td>Immediate cooling to ambient temperature</td>
<td>Extended time for dissipation of heat due to packaging (25 kg bags or 1 ton totes)</td>
</tr>
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**Figure Legends**

**Fig. 1.** Group mean anti-PCV2-IgG sample-to-positive (S/P) ratios and standard error results for pigs intraperitoneally inoculated with spray-dried plasma (SDP-IP), pigs orally gavaged with PCV2-infected spray-dried plasma (SDP-OG), pigs intraperitoneally inoculated with PCV2-positive plasma (POS) or pigs which were non-inoculated (NEG). Group means and standard errors were calculated using all three animals in each group at each time point. S/P ratios equal or greater than 0.2 are considered positive. Significant different group means at a given time point are indicated by different superscripts (A, B).
Fig. 2. Group mean log$_{10}$ PCV2 genomic copies per ml serum and standard errors for pigs intraperitoneally inoculated with PCV2-infected spray-dried plasma (SDP-IP), pigs orally gavaged with PCV2-infected spray-dried plasma (SDP-OG), pigs intraperitoneally inoculated with PCV2-positive plasma (POS) or pigs which were non-inoculated (NEG). Group means and standard errors were calculated using all three animals in each group at each time point. Significant different group means at a given time point are indicated by different superscripts (A, B).
CHAPTER 6. Disinfection protocols reduce the amount of porcine circovirus type 2 (PCV2) in contaminated livestock transport vehicles

A paper submitted to the *Journal of Swine Health and Production*

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ABSTRACT

**Objective:** To evaluate the ability of four disinfection methods to reduce porcine circovirus type 2 (PCV2) in livestock trailers. **Materials and Methods:** Trailer models were contaminated with intestinal content collected from a pig experimentally infected with PCV2, washed, and then disinfected with one of four disinfection protocols. Between each step, six swabs of each trailer were taken and tested by PCV2 PCR. The disinfection protocols were: DISF1 (quaternary ammonium compound), DISF2 (oxidizing agent containing potassium peroxomonosulfate), DISF3 (combined glutaraldehyde and quaternary ammonium product) and DISF4 (DISF2 followed by a sodium hypochlorite compound). Also included were PCV2 contaminated, non-washed, non-disinfected trailers (POS control treatment) and an uncontaminated trailer (NEG control treatment). Two PCV2-naïve pigs were placed in each trailer for two hours. Blood samples were collected weekly for seven weeks and tested for the presence of anti-PCV2-IgG antibodies and PCV2 DNA. **Results:** In all four disinfection protocols the initial wash step significantly (P<0.05) reduced the mean amount of PCV2 DNA present compared to the POS control treatment; however, only DISF4 (P<0.0001) reduced the mean amount of PCV2 DNA compared to the amount present after the wash.
step. After disinfection, between 1.5 and 5.2 \( \log_{10} \) PCV2 genomic copies/ml were identified in the trailers. While animals exposed to POS control trailers became viremic and seroconverted, no seroconversion or viremia was detected in animals exposed to disinfected trailers. **Implications:** Although PCV2 DNA was detected in all trailers after disinfection, PCV2 was not transmitted to naïve animals exposed to the trailers under the study conditions.

Keywords: Porcine circovirus type 2, transmission, transportation, swine

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, circular, single stranded DNA virus that was first isolated from pigs exhibiting multisystemic wasting disease in the late 1990’s.\(^1\) Since the initial description, PCV2-associated disease (PCVAD) has been reported in most pig producing countries worldwide and the virus is now considered ubiquitous.\(^{18}\) PCVAD is now manifested in a variety of ways including multisystemic disease with wasting, reproductive failure, enteritis, respiratory disease, porcine dermatitis and nephropathy syndrome or a combination of these.\(^{15}\) In cases of PCVAD, a morbidity rate of 4-30% with a mortality rate of 70-80% in affected animals has been reported.\(^{20}\) Commercial PCV2 vaccines are now available and have generally been very effective in reducing morbidity and mortality in the field\(^5\) and under experimental conditions.\(^6\)

PCV2 has been shown to be shed in respiratory secretions, oral secretions, urine and feces.\(^{20}\) It is a hardy virus that is quite stable in the environment. In an *in vitro* study\(^{12}\), PCV2 virus stock was subjected to various heat treatments ranging from 56°C to 85°C for 15 min. Following incubation, the virus stock was added to PK-15 cells and evidence of PCV2
infection was documented. Under these conditions, PCV2 antigen staining was not detected at treatment temperatures between 75°C to 85°C although PCV2-PCR products were identified in samples treated at 75°C. In other studies, dry-heat treatment of freeze-dried PCV2 at 120°C for 30 min resulted in only a 1 log reduction of the virus and PCV2 retained an infectivity titer of $1 \times 10^{2.7}$ following exposure to a pH of 2 for 30 min and a titer of $1 \times 10^{2.5}$ following incubation at 70°C for one hour.

Previous work also suggests that under in vitro conditions, mean PCV2 titers were reduced but not completely eliminated following exposure to various disinfectants with oxidizing products and aldehyde/quaternary ammonium combination products showing the highest efficacy. In a separate study, exposure of PCV2 cell suspensions to an oxidizing product or to a sodium hydroxide compound led to a complete reduction in PCV2 infectivity following a 10 min incubation period. However, neither a cytotoxicity control nor a virus control (virus plus media alone) were used in this study. While the above information exists on the effectiveness of disinfectants under in vitro conditions, to the authors’ knowledge no information exists on the effectiveness of disinfectants under field conditions.

Knowledge of the modes of transmission of PCV2 is vital in understanding the ecology, epidemiology and control of PCVAD. Specific information on the horizontal transmission of PCV2 via fomites such as commercial livestock transport trailers is lacking. The increased use of multi-site production facilities has led to an increased frequency of animal movement and with this there is an increased risk of pig exposure to pathogens while in transport. To the authors’ knowledge, no information exists to date on the ability of commercial transport
trailers to aid in transmission of PCV2 or on the ability of various disinfectant protocols to decrease or eliminate the risk of transmission of PCV2 via this route. The objective of this study was to evaluate the ability of four disinfection protocols with known in vitro effectiveness against PCV2 to prevent transmission of PCV2 under simulated field conditions. To accomplish this, the amount of PCV2 DNA present in PCV2 contaminated, washed, disinfected trailers (DISF1-4 treatments) was compared to the amount of PCV2 DNA present in a PCV2 contaminated, non-washed, non-disinfected trailer (POS control treatment). In order to determine the infectivity of the PCV2 DNA detected by PCR, naïve pigs were exposed to trailers with either DISF1-4 treatments, POS control treatments, or an uncontaminated trailer (NEG control treatment).

MATERIALS AND METHODS

Experimental design

The experimental protocol in this study was approved by the Iowa State University Institutional Animal Care and Use Committee. The first objective of the study was to determine if the disinfection step reduced the amount of PCV2 present on the trailer surface. To accomplish this, trailer models (experimental unit) were contaminated with fecal material collected from a pig experimentally infected with PCV2 and showing clinical signs consistent with PCVAD (n=11) or remained uncontaminated (n=1). Six swabs (subsamples taken from six different trailer surface locations, Figure 1 B-G) were taken of each trailer following contamination, designated as “Post-contamination” samples and tested for the presence of PCV2 DNA by real-time PCR. Following PCV2 contamination, positive control trailers (n=4) were not washed. All other trailers (n=7) were washed. Six swabs of
each of the washed trailers were taken, designated as “Post-wash” and tested by real-time PCV2 PCR. Following the wash step, the trailers were disinfected with one of four disinfection protocols (DISF1-4). Six swabs of each of the disinfected trailers were taken, designated as “Post-disinfection” and tested by PCV2 real-time PCR. A summary of the number of replicates and swabs taken at each sampling point is outlined in Table 1. High numbers of replicates of the POS control treatment were done to gain a statistically more precise estimate of a subset of the collected data (post-contamination vs. post-wash, and post-contamination vs. post-disinfection).

The second objective of the study was to determine whether the PCV2 DNA present in disinfected trailers (DISF1-4) was infectious to naïve animals. To answer this question, a total of 24 PCV2 naïve pigs were used by placing pigs in groups of two into trailers which were randomly assigned to a treatment (POS control, n=8 pigs; NEG control, n=2 pigs; or DISF1-4, n=14 pigs). The pigs were exposed to the trailers for a total of two hours. After this, the pigs were moved to the research facility and were kept by group in rooms for 49 days following trailer exposure. The pigs were monitored on a weekly basis for development of anti-PCV2-antibodies by ELISA and PCV2 viremia by real-time PCR.

For this portion of the study, an incomplete block design with three experimental units (trailers containing two pigs) per block, four blocks, and six treatments was used (Table 2.). Within each block, the trailer was considered the independent experimental unit and treatments were randomly assigned to trailers. Each block included a POS control treatment and two of the remaining five possible treatments (DISF1-4 or NEG control). There were two
replicates each for the DISF1, DISF2, and DISF3 treatments. There was one replicate each of the NEG control and DISF4 treatments because those treatments were expected to be the most different from the POS control based on previous in vitro work. Blocks were included in the study to account for variation in environmental conditions.

PCV2 contaminated fecal material

A colostrum-fed, crossbred, specific-pathogen-free, PCV2-naïve pig was inoculated with 5 ml (3 ml intranasally and 2 ml intramuscularly) of $10^{4.0}$ 50% tissue culture infectious dose (TCID$_{50}$) per ml of PCV2b isolate NC-16845 (accession no. EU340258) at 3 weeks of age. At 35 days post inoculation, the pig was euthanized with an overdose of pentobarbital (Fatal Plus®, Vortech Pharmaceutical, Dearborn, Michigan, USA) due to the development of clinical signs consistent with PCVAD including severe dyspnea, diarrhea, and loss of condition. After euthanasia, the complete contents of the small intestine, cecum and colon were collected. Twenty ml aliquots of intestinal contents were transferred into 50 ml centrifuge tubes and stored at -20°C until use. A portion of the fecal material was tested for the presence of PCV2 DNA by real-time PCR; 10.8 log$_{10}$ genomic copies/ml of PCV2 DNA were recovered. PCV2 systemic infection of the animal was further confirmed by the presence of intense PCV2 antigen staining by immunohistochemistry (IHC) in the lungs, intestine and lymphoid tissues. Transmissible gastroenteritis virus antigen, rotavirus type 2 antigen, and Lawsonia intracellularis antigen were not detected by IHC. Porcine reproductive and respiratory syndrome virus (PRRSV) RNA, swine influenza virus (SIV) RNA, and Mycoplasma hyopneumoniae DNA were not detected by PCR. All of the tests were
performed following the standard operating procedures for the specific tests at the Iowa State University Veterinary Diagnostic Laboratory.

**Trailers**

For each block, three model trailers were used to evaluate three of the six treatments. Trailers were designed and constructed by a company specializing in standard and custom aluminum livestock transportation equipment (EBY Inc., Story City, Iowa, USA). The trailer models were specifically designed to accurately portray a standard semi-livestock trailer used in the transportation of commercial swine. The models were composed of 29 mm thick 6061 T-6 aluminum alloy diamond plate flooring welded and riveted to tubular extruded aluminum cross-members, 19 mm thick aluminum smooth sheet side walls with both large (25.4 cm × 14.9 cm) and small (8.9 cm × 7.0 cm) ventilation holes with an overlapped seam at the side post, extruded aluminum side posts with integral closure track, a hinged aluminum rear door, a center divide gate and a simulated roll-up door (Figure 1). Final dimensions were 0.62 m (width) × 0.82 m (height) × 1.11 m (length). The trailer models were 1:61 scale models of a full sized trailer and had a floor space of 0.69 m² which corresponded to 41.85 m² (2.59 m wide and 16.16 m long) for the full-sized trailer, and a constant animal density of 0.34 m² (trailer floor space in m² divided by number of animals per trailer).

**Trailer sampling protocol**

At each of the four sampling points (pre-contamination, post-contamination, post-wash, post-disinfection) six swabs (polyester-tipped swab, Fisher Scientific Inc., Pittsburgh, Pennsylvania, USA) were taken of each trailer (Figure 1, B-G). Each swab was placed into 1
ml of sterile saline (0.9% sodium chloride solution, Fisher Scientific Inc.), used to swab a 5.5 cm diameter area (using a template) and then returned for storage at -80°C in a 5 ml plastic tube (BD falcon tube, Fisher Scientific Inc.) prior to testing by PCR. To prevent carryover contamination between trailers during a certain sampling step, a new template was used for each trailer, and new gloves were used by the investigator. To prevent carryover between sampling steps, templates were submerged in an oxidizing disinfectant (Virkon®S, DuPont Chemical Solutions Enterprise, Wilmington, Delaware, USA) for 10 min between sampling points.

**Trailer contamination protocol**

To contaminate each of the trailers (except the trailer used for the NEG control treatment), a 20 ml aliquot of the PCV2 contaminated fecal material was thawed at 4°C overnight and spread on the walls and floor of the trailer by the same investigator for each trailer within a block. Following contamination, 200 g of bedding (woodchips; Theisen’s, Ames, Iowa, USA) were placed on the floor of the trailer. In order to maintain similar experimental conditions between groups bedding was also placed in the trailer assigned to the NEG control treatment.

**Trailer washing protocol**

To prevent cross-over contamination from the previous step, investigators changed gloves prior to the wash step. The trailers were washed using the following protocol: The trailer was first rinsed using a low pressure (50 psi) nozzle to flush out the majority of woodchips and gross debris, then washed with 48.9°C water using a high-pressure (2400 psi) nozzle for
approximately 30 sec and last rinsed using a low pressure nozzle to remove any remaining gross debris.

**Trailer disinfection protocol**

To prevent cross-over contamination from the previous step, a new investigator or an investigator who had showered and changed clothes and gloves applied disinfectant to the trailer. Gloves were changed between trailers and ample room was available to prevent cross-over of disinfectants between trailers within a block. Disinfectants were applied using a liquid concentrate sprayer (H.D. Hudson Manufacturing Company, Chicago, Illinois, USA) attached to a garden hose. The following disinfectants which correspond to the experimental design in Table 1 were used: DISF1, a quaternary ammonium compound (PI Quat 20®, Preserve International, Reno, Nevada, USA); DISF2, an oxidizing agent containing a potassium peroxomonosulfate (Virkon®S); DISF3, a combined glutaraldehyde and quaternary ammonium product (Synergize®, Preserve International, Reno, Nevada, USA); and DISF4, an application of two disinfectants in tandem, the first being the previously described oxidizing agent (Virkon®S,) followed by a chloride compound (Clorox® Bleach, The Clorox Company, Oakland, California, USA). Disinfectants were applied at the following rates based on the respective manufacturers’ recommendations: DISF1 was applied at a rate of 59 ml/18.9 liter (2 oz/5 gal) of water, DISF2 was reconstituted to a concentration of 100 g/liter and applied at a rate of 30 ml/3.8 liter (1 oz/gal) water, and DISF3 was applied at a rate of 15 ml/3.8 l (0.5 oz/gal) water. For DISF4, the oxidizing agent was applied at the previously described rate and the chloride compound was applied at a rate of 177.4 ml/3.8 liter (6 oz/gal) of water. Disinfectants were applied and after 10 min of contact time the
trailers were rinsed with water. The temperature through the disinfectant process was similar (20°C) for all blocks.

**Decontamination protocol between blocks**

To ensure that residual contamination of the trailer between the four blocks did not occur, trailers were washed with 48.9°C water using a high-pressure (2400 psi) nozzle, scrubbed with a detergent product (Ultra Palmolive® OXY® plus with Bleach Alternative, Colgate-Palmolive Company, New York, New York, USA), thoroughly rinsed, disinfected with an oxidizing agent (Virkon®S) and allowed to dry completely. This process was repeated until PCV2 DNA was not detected by real-time PCR from six different trailer surface locations (Figure 1, B-G) by using swabs.

**Animals**

Blocks no. 1 and no. 2 were performed with animals from source A in September 2008 and blocks no. 3 and no. 4 were performed using pigs from source B in February 2009. Obtaining pigs from two sources was not part of the study design, but it was necessary due to the inability to obtain PCV2 naïve animals from source A in February of 2009. The use of blocks accounted for the variability introduced from using two pig sources. In both sources, pigs were colostrum-fed, crossbred, specific-pathogen-free conventional pigs purchased from herds that were routinely tested for major swine pathogens and known to be free of PCV2, PRRSV, porcine parvovirus and SIV. Source A pigs were weaned at three weeks of age and raised to approximately 18-27 kg at which time they were transported to the Livestock Infectious Disease Isolation Facility (LIDIF) at Iowa State University, Ames, Iowa. Source B
pigs weaned at two weeks of age and raised to approximately 10-22 kg at which time they were transported to the LIDIF. Pigs were housed in groups of two in separate rooms. Each room had 18 m² of solid concrete floor space, separate ventilation systems and one nipple drinker. All groups were fed a balanced, pelleted, complete feed ration free of animal proteins (excluding whey) and antibiotics (Nature’s Made, Heartland Coop, Iowa, USA) once a day. Pigs were tested upon arrival at the facility by real-time PCV2 PCR\textsuperscript{17} and PCV2 ELISA\textsuperscript{11} on serum to confirm that they were PCV2 free prior to initiation of the trial.

**Exposure of the pigs to the trailer models**

Following disinfection, the three model trailers were placed into a clean equine trailer (no prior exposure to swine) and separated by clear polyethylene sheeting. Two pigs were placed in each trailer for a total exposure time of two hours. During this time, the trailers were driven on a specific route which included approximately 32.2 km of interstate, 56.3 km of state highway, and 32.2 km unpaved (gravel) road through Hardin, Story and Hamilton counties in the state of Iowa. Following the two hour exposure period, pigs were placed in isolation rooms by treatment group. At the time of block no. 1, the temperature was 22.8°C and it rained throughout the time of transport. At the time of block no. 2, the temperature was 29.4°C with no precipitation. At the time of block no. 3, the temperature was 1.1°C with no precipitation. At the time of block no. 4, the temperature was -12.2°C with no precipitation.

**Anti-PCV2-IgG antibodies and PCV2 DNA quantification and sequencing**

Blood samples were collected on the day of exposure, and weekly thereafter until 49 days post trailer exposure. The blood was collected in 8.5 ml serum separator tubes (Fisher
Scientific Inc., Fair Lawn, New Jersey, USA), immediately centrifuged at 2000 $x$ g for 10 min at 4°C and stored at -80°C until use. Serum samples were tested by an ORF2-based PCV2 IgG ELISA and were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater as previously described. DNA-extraction on serum samples was performed using the QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, California, USA). Previously described primers for ORF 1 of PCV2$^{17}$ were used for quantification of the PCV2 genomic DNA by real-time PCR. The PCR reaction consisted of 25 µl PCR mixtures that contained 12.5 µl of commercially available master mix (TaqMan Universal PCR Master Mix, Applied Biosystems Inc., Foster City, California, USA), 2.5 µl DNA extract, 1 µl forward and reverse primers, and 0.5 µl detection probe with concentrations of 10 µM. On each plate five progressive 1:10 dilutions of a known copy number of PCV2 genomic DNA excised from a purified PCV2 DNA clone was included to generate a standard curve. Each plate was run in the sequence detection system (7500 Sequence Detection System; Applied Biosystems Inc.) under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Samples which did not generate a signal following 40 cycles were considered negative. PCR products amplified from virus recovered from a serum sample from one animal in each of the positive control groups, was sequenced and compared with the PCV2 in the fecal material used to contaminate the trailers. A nested PCR was used to amplify the entire ORF2 gene for sequencing as previously described.$^{14}$ PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc.) per the manufactures directions and sequenced at Iowa State University DNA facility. Sequences were analyzed with Sequence Scanner 1.0 (Applied Biosystems Inc.) and compared with the sequence of the PCV2 present in the fecal material using the basic local alignment search
Necropsy

All pigs were necropsied at 49 days post trailer exposure. Severity of macroscopic lung lesions ranging from 0 to 100% of the lung affected were estimated and the size of lymph nodes was scored from 0 (normal) to 3 (four times the normal size) in a blinded fashion as described previously. Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy and fixed in 10% neutral-buffered formalin and routinely processed for histological examination.

Histopathology

Microscopic lesions were evaluated by a pathologist blinded to treatment groups. Lung sections were scored for the presence and severity of interstitial pneumonia ranging from 0 (normal) to 4 (severe interstitial pneumonia) as described previously. Sections of heart, liver, kidney, ileum and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues including lymph nodes, tonsil, and spleen were evaluated for the presence of lymphoid depletion ranging from 0 (normal) to 3 (severe) and histiocytic inflammation and replacement of follicles ranging from 0 (normal) to 3 (severe).

Immunohistochemistry (IHC)

IHC for detection of PCV2-specific antigen was performed on selected formalin-fixed and
paraffin-embedded sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, and mesenteric), tonsil, spleen, Peyer’s patches, and thymus using a rabbit polyclonal antiserum as described previously.\textsuperscript{21} PCV2-antigen scoring was done by a pathologist blinded to treatment groups. Scores ranged from 0 (no signal) to 3 (more than 50\% of the lymphoid follicles contain cells with PCV2-antigen staining).\textsuperscript{16}

**Statistical analysis**

The statistical analysis for objective 1, to determine if disinfection reduced the amount of PCV2 present on the trailer surface, focused on the differences in log genomic copies of PCV2 between three steps of each treatment protocol (post-contamination, post-washing, and post-disinfection) and between four disinfectants. The experimental unit was the trailer within a block. The assumption of independence of experimental units was reasonable because each trailer was carefully cleaned to eliminate carryover between blocks. The amount of PCV2 DNA was measured on six swabs (subsamples) at each step within an experimental unit; these were averaged before statistical analysis.

Trailers assigned to DISF1-4 treatments were sampled at four steps of the decontamination process (pre-contamination, post-contamination, post-washing, and post-disinfection). Trailers assigned to the POS control treatment were sampled only twice (pre-contamination and post-contamination). Trailers assigned to the NEG control treatment were sampled only once. Data from the pre-contamination step and the NEG control treatment were excluded from the analysis because no PCV2 DNA copies were detected. The POS control and DISF1-4 treatments were used to define three unique sampling steps: post-contamination (POS...
control treatment; DISF1-4), post-washing (DISF1-4 combined), and individual post-disinfection treatments (DISF1, DISF2, DISF3, or DISF4). This is appropriate because after contamination (prior to washing) all trailers were treated alike and after washing (prior to disinfection) all washed trailers were treated alike.

The statistical analysis accounted for correlation between the three repeated measurements on the disinfected trailers (post-contamination, post-washing, and post-disinfection) by including a random effect for the experimental unit. Data was analyzed using PROC MIXED in SAS version 9.2 (SAS Institute, Inc., Cary, North Carolina, USA). The variation in environmental temperatures was accounted for by including block as a fixed effect in the model. Variance components were estimated using REML and the Satterthwaite approximation was used to compute degrees of freedom. The type 3 F test of treatments was considered significant if the p-value was < 0.05. Differences of least squares means using the Tukey-Kramer adjustment were evaluated if the F test was significant.

There was no statistical evaluation for objective 2 of the experiment as neither seroconversion nor viremia was detected in any of the animals exposed to disinfected (DISF1-4) trailers.

**RESULTS**

**Amount of PCV2 DNA was reduced on the trailer surfaces following the wash step**

The mean amounts of PCV2 DNA (log_{10} genomic copies/ml) obtained for each of three sampling points (post-contamination, post-wash, and four levels of post-disinfection) are
presented in Table 1. The F-test of equal treatment means was highly significant (P<0.0001). Differences of least squares means using the Tukey-Kramer adjustment indicated a significant reduction in mean log₁₀ genomic copies/ml of PCV2 DNA between the post-contamination step and the post-wash step. Only the DISF4 protocol significantly (P<0.0001) reduced the mean amount of PCV2 DNA present compared to that present after the post-wash step.

**Evidence of PCV2 infection was not present in naïve animals exposed to contaminated, disinfected trailers**

Clinical disease was not observed in any of the pigs for the duration of the study. Seroconversion to PCV2 was not detected in the any of the animals exposed to the NEG control, DISF1, DISF2, DISF3, or DISF4 treatments during any of the replicates. Both positive control pigs seroconverted by 28 days post trailer exposure in blocks no. 1, 2 and 4. In block no. 3, one positive control pig seroconverted by 14 days post trailer exposure and the other by 42 days post exposure. Viremia was not detected in any of the animals exposed to trailers treated with DISF1, DISF2, DISF3, or DISF4 protocols or the NEG control treatment during any of the replicates. Both pigs in the POS control group became PCV2 viremic by 21 days post exposure in blocks 1, 3, and 4. Both POS control pigs in block no. 2 became PCV2 viremic by 14 days post exposure. Characteristic microscopic lesions of PCV2 infection or PCV2 antigen staining were not noted in any of the animals from the NEG control, DISF1, DISF2, DISF3, or DISF4 groups. In POS control pigs (n=8), there was mild to moderate depletion of follicles in the tonsil (1/8) and in lymph nodes (5/8). Histiocytic replacement of lymphoid germinal centers was observed in 2/8 animals. Low to moderate amounts of PCV2 antigen were detected in the tonsil of 4 of the 8 pigs and in lymph nodes of
6 of the 8 pigs. Mild lymphoplasmacytic myocarditis, hepatitis and interstitial nephritis were noted in 3/8, 2/8 and 3/8 animals, respectively.

**DISCUSSION**

The overall objective of this study was to evaluate the ability of four disinfection protocols with known *in vitro* effectiveness against PCV2 to prevent transmission of PCV2 under simulated field conditions. Previous work has shown oxidizing agents, aldehyde compounds and quaternary ammonium compounds have efficacy against PCV2 under *in vitro* conditions.\(^9,10,19\) While *in vitro* efficacy data is useful, field efficacy of a disinfectant depends on the type of surface and the presence of organic material and other variables.\(^1\) To evaluate the effectiveness of the disinfection protocols under simulated field conditions, PCR analysis of trailers following the contamination, wash and disinfection steps was performed to assess whether virus was present following each of the steps in the protocols. Second, the infectivity of the PCV2 DNA detected on the trailer surface was evaluated by exposing naive animals to the trailers for two hours.

The results of the study indicated that the wash step significantly reduced the amount of PCV2 DNA present in the trailers in comparison to the POS control treatment. However, following the disinfection step, between 1.5 and 5.2 log\(_{10}\) PCV2 genomic copies/ml were identified in the DISF1-4 trailers. This is in agreement with previous *in vitro* work which found that mean PCV2 titers were reduced but not completely eliminated following exposure to various disinfectants.\(^10,19\) Interestingly, washing the trailer alone significantly reduced the amount of PCV2 DNA present in the trailers and there was not a significant difference
between the washing and disinfection steps when only one disinfectant was applied (i.e. excluding DISF4 protocol where two disinfectants were applied). This supports the principle that thorough removal of organic material prior to application of a disinfectant is essential to the cleaning process. While it is possible that the small number of replicates performed for each of the disinfectants hindered the ability to detect a difference between the washing and disinfectant steps, a significant difference was detected in the disinfectant protocol with the least number of replicates (DISF4). In regards to the significant reduction of PCV2 DNA noted in the DISF4 protocol, it is unknown whether the combination of the two disinfectants or just the additional rinsing associated with the application of two disinfectants was responsible for the outcome and additional studies in this area are warranted.

The detection of PCV2 in trailers following disinfection does not necessarily correlate with transmission as detection of virus by PCR does not confirm that the virus is infectious. While virus isolation could have been performed to determine the infectivity of the detected PCV2, it is documented that PCV2 grows slowly, requires optimized culture conditions and that virus isolation is not always successful. Additionally, viral isolation of PCV2 from rectal swabs in experimentally infected cesarean-derived, colostrum-deprived pigs showing clinical signs of PCVAD was infrequent even though virus isolation from serum was successful up to five weeks post-infection. Therefore, we elected to do a bioassay where naïve animals were exposed to the trailers.

To accurately simulate field conditions, 1:61 scale model trailers were designed and manufactured by a company specializing in standard and custom aluminum livestock
transportation equipment with the exact materials used during commercial production. Trailers were manufactured purposely to contain areas where fecal material would likely accumulate and sampling of the trailers was concentrated on these areas to accurately determine viral concentration within the trailer following each step in the protocol.

Interestingly, the thorough disinfection protocol used on trailers between blocks to prevent carryover contamination needed to be performed multiple times, especially in block no. 3 and no. 4 where environmental temperatures prevented exposure of the trailers to natural ultraviolet light. This confirmed that the sampling areas were able to harbor PVC2 DNA. By design, the model trailers used in the study provided adequate space to allow animals to turn completely around. This allowed for increased exposure to areas where contaminated feces may have accumulated. Although effort was made to ensure similarity with field conditions, changes associated with normal wear of a trailer were not replicated as trailers were manufactured from new materials and used for the first time during this trial.

Fecal material from one experimentally infected animal at the peak of infection was used in this study to provide a consistent, high amount of virus in each trailer (10.8 log_{10} PCV2 genomic copies/ml). All pigs in the POS control trailers seroconverted and became viremic indicating that in each of the blocks the virus was infectious to naïve animals. Previously published information suggests the presence of approximately 2 to 6 log PCV2 genomic copies/ml in fecal samples from clinically healthy animals, animals with mild PCV2 associated lesions and animals with moderate to severe microscopic lesions associated with PCVAD. Based on the above data, the concentration of virus used in this study was above what would be expected under field conditions.
During the study, naïve animals were exposed to the trailer models for two hours and transported over paved and unpaved roads in counties in Iowa with high densities of swine operations. This contact time is similar to trials done with PRRSV and is representative of transport of pigs between local geographic areas. However, the study is not representative of transport between distant geographic areas in which animals may be transported for 12 to 20 hours. Examples of this would include transport of weaned piglets from farrowing facilities in Canada or North Carolina to finisher facilities in the Midwest. As $1.45 \pm 0.6 \log_{10}$ genomic copies of PCV2 remained in the trailer following the most efficacious disinfectant protocol, it is possible that longer exposure periods could have resulted in transmission of PCV2 to naïve animals.

Great effort was made to ensure washing and disinfectant application was similar to field conditions. Specifically, a protocol (D. Miller, personal communication) similar to one used in a commercial truck wash facility including a low pressure rinse, a high pressure wash and a final low pressure rinse to remove gross debris was followed. However, in commercial truck wash facilities worker speed to ensure a certain number of trailers per hour are cleaned may lead to less diligence toward the cleaning and disinfection process in comparison to the experimental conditions of this study.

In this study, PCV2 was not transmitted to naïve animals after application of any of the four disinfection methods even though the following conditions were true: (1) naïve animals were exposed to a concentration of infectious virus above that which has been reported from fecal
samples of naturally infected animals, (2) naïve animals had more than the typical amount of floor space in the trailer allowing increased movement and exposure to fecal material containing virus, and (3) model trailers were designed to include places in which feces are typically difficult to thoroughly remove. However, the number of animals used was low. The upper 95th percentile for the probability of infection \( P_{\text{infect}} = \exp(\ln(1 - P_{\text{detect}}) = N) \) where \( N = \) the number of animals in each replicate and \( P_{\text{detect}} = 0.95 \) was 0.53 and 0.78 for DISF1-3 and DISF4, respectively.

Implications

- Washing the trailer with water alone significantly reduced the amount of PCV2 DNA present in the trailers.
- There was no significant difference between the washing and disinfection steps when only one disinfectant was applied.
- DISF4 (DISF2 oxidizing agent followed by a sodium hypochlorite compound) treatment significantly (\( P<0.0001 \)) reduced the mean amount of PCV2 DNA compared to the amount present after the wash step.
- Although between 1.5 and 5.2 \( \log_{10} \) PCV2 genomic copies/ml remained in the trailer models after disinfection, under the conditions of this study PCV2 was not transmitted to naïve animals.

ACKNOWLEDGEMENTS

The authors would like to thank Paul Thomas, Jeremy Johnson and the laboratory animal resources staff for assistance with animal work; Erin Varley, Don Miller and Mitch
Shellenberger for assistance in design and manufacturing of the trailer models; Philip Dixon for statistical assistance and the National Pork Board for funding of the study through the Pork CheckOff dollars.

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14. Opriessnig T, McKeown NE, Zhou EM, Meng XJ, Halbur PG. Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without


**Table 1:** Mean log_{10} genomic copies/ml of PCV2 DNA from each of the three sampling points (post-contamination, post-wash, post-disinfection). Data from swabs taken from the post-contamination step were considered in the same group regardless of which disinfection step it was followed by. A similar approach was used for data from the post-wash step. Post-disinfection samples were considered independently for each disinfection protocol (DISF1-4). The number of replicates and swab samples used in the statistical analysis are provided in the table.

<table>
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<tr>
<th>Step</th>
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</table>

^ Different superscripts (a,b,c) within a column indicate significant (P<0.05, Tukey-Kramer adjusted t-value for differences of least squares means) differences among steps.

^†DISF1, a quaternary ammonium compound (PI Quat 20®, Preserve International, Reno, Nevada, USA)

^‡DISF2, an oxidizing agent containing a combination of potassium peroxymonosulfate and sodium chloride (Virkon®S, DuPont Chemical Solutions Enterprise, Wilmington, Delaware, USA)

^∫DISF3, a combined glutaraldehyde and quaternary ammonium product (Synergize®, Preserve International, Reno, Nevada, USA)

^δDISF4, an application of two disinfectants in tandem, the first being an oxidizing agent (Virkon®S, DuPont Chemical Solutions Enterprise, Wilmington, Delaware, USA) followed by a chloride compound (Clorox® Bleach, The Clorox Company, Oakland, California, USA)
**TABLE 2:** Incomplete block design using three trailers and four replicates to determine whether PCV2-naïve animals would become PCV2 seropositive or viremic following a 2 hr. exposure period to PCV2 contaminated, non-washed, non-disinfected trailers (POS control treatment); PCV2 contaminated, washed, disinfected trailers (DISF1-4 treatment) or uncontaminated trailers (NEG control treatment).

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<tr>
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<td>NEG</td>
<td>POS</td>
</tr>
<tr>
<td>2</td>
<td>POS</td>
<td>DISF2†</td>
<td>DISF1</td>
</tr>
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<tr>
<td>4</td>
<td>POS</td>
<td>DISF3</td>
<td>DISF4 δ</td>
</tr>
</tbody>
</table>

*DISF1, a quaternary ammonium compound (PI Quat 20®, Preserve International, Reno, Nevada, USA)
†DISF2, an oxidizing agent containing a combination of potassium peroxymonosulfate and sodium chloride (Virkon®S, DuPont Chemical Solutions Enterprise, Wilmington, Delaware, USA)
‡DISF3, a combined glutaraldehyde and quaternary ammonium product (Synergize®, Preserve International, Reno, Nevada, USA)
δDISF4, an application of two disinfectants in tandem, the first being an oxidizing agent (Virkon®S, DuPont Chemical Solutions Enterprise, Wilmington, Delaware, USA) followed by a chloride compound (Clorox® Bleach, The Clorox Company, Oakland, California, USA)
Figure 1: Trailer models designed as 1:61 scale models of a full sized standard semi-livestock trailer used in the transportation of commercial swine (A). The models were composed of aluminum smooth sheet side walls welded and riveted to tubular extruded aluminum cross-members (B), a hinged aluminum simulated roll-up rear door (C), aluminum alloy diamond plate flooring (D), a latch (E), an overlapped seam at the side post (F) and center divide gate (G). Final dimensions were 0.62 m width × 0.82 m height × 1.11 m length.
CHAPTER 7. Establishment and maintenance of a porcine circovirus type 2 (PCV2)-free research breeding herd on a site that experienced a natural outbreak of PCV2-associated reproductive disease

A paper submitted to the Journal of Swine Health and Production

Abby R. Patterson, Darin M. Madson, Shayleen D. Harrison, Pat G. Halbur, Tanja Opriessnig

ABSTRACT

The objectives were to document a porcine circovirus type 2 (PCV2)-associated reproductive failure outbreak in a closed, PCV2-naive specific pathogen free herd in Iowa in 2009, to describe the elimination of infectious PCV2 from the breeding herd site, to report the outcome after repopulation, and to attempt to derive PCV2 negative animals by offsite segregation. Clinical signs were limited to an increased incidence of mummified fetuses. After confirmation of PCV2-associated lesions in the fetuses and PCV2 viremia in dams, the herd was depopulated. Cleaning and disinfection of the premise prior to repopulation included removal of gross organic material, exposure of equipment to natural UV light, multiple applications of disinfectant, and application of paint or sealer to porous surfaces. During the 63 day clean-up period, no pigs were on the site. An improved biosecurity plan
was also implemented. The herd was repopulated and a PCV2-naïve population has since been maintained at the facility to date (>300 days). Attempts to derive PCV2 negative pigs from the positive herd following offsite segregation were unsuccessful. The combination of a multistep cleaning and disinfection protocol with a strict biosecurity plan can result in the maintenance of PCV2 naïve animals on a previously contaminated site.

Key words: Porcine circovirus type 2, swine, disinfection, biosecurity

Porcine circovirus type 2 (PCV2) is a small, non-enveloped, single-stranded DNA virus which emerged in the 1990’s as an economically important swine pathogen.6 The first report of PCV2 reproductive disease occurred in a commercial 450-head herd composed entirely of first parity dams.38 The timing of infection was not determined in that case and clinical signs on the farm included late-term abortions (losses after 13 weeks of gestation), decreased farrowing rates and increased stillborns and mummified fetuses; no clinical signs were reported in the gilts. In one of nine examined fetuses, myocarditis and abundant PCV2 antigen was identified in fetal myocardium. Other common pathogens associated with reproductive disease were not detected.38 Multiple case reports on PCV2-associated reproductive failure have subsequently been described in seropositive herds8,22 or in start-up herds or gilt populations which may have been serologically naïve to PCV2.2,19,20,29 Common clinical features included increased numbers of mummified fetuses and stillborns at parturition, none or low numbers of dams exhibiting clinical signs, and resolution of clinical signs between two to five months following initial detection.
PCV2 is quite stable in vitro\textsuperscript{24,37} and many disinfectants do not completely eliminate the virus under in vitro conditions\textsuperscript{11,17,31}. To our knowledge, no documented information exists on facility disinfection procedures for establishment and maintenance of a PCV2 negative herd. However, a procedure for room decontamination used successfully by researchers at Iowa State University to disinfect rooms between PCV2-animal inoculation studies was recently described.\textsuperscript{5}

The objectives of the current study were to document a natural outbreak of PCV2 in a high health population previously known to be free of PCV2, to describe the procedure used to disinfect the premise, report the outcome after repopulation, and document an attempt to derive PCV2 negative animals by offsite segregation.

**GENERAL INFORMATION**

*Building descriptions*

The source herd was housed in a facility located in Iowa originally built in the 1970’s. The facility is composed of five 22.8 m × 15.2 m buildings in close proximity (15-30 m) to each other with an associated waste lagoon (Figure 1). An uncovered concrete walkway (Figure 1) connects the individual buildings and was installed after herd depopulation following the 2009 outbreak.

*General building:* Building 1 is used as the general services building which is divided into two sides by a concrete wall. Side one is used as the entrance to the facility with office space, shower, laundry and locker room. Side two is used as storage for feed and equipment and
tools for building maintenance. Building 1 has one door for entry into the sign-in area, a door that leads out from the office area and a garage door for entry into a feed and equipment storage area.

*Animal buildings:* Buildings designated 2 through 5 are used for animal housing. The animal buildings are completely enclosed, power ventilated, and partially slatted with pull-plug drains. Animal buildings 2, 3, and 5 have a center concrete wall which divides the building into two halves; each half has a separate access point. Building 2, 3, and 5 have each two manure pits which are 1.2 m wide, 3.4 m long and 0.9 m deep. Building 4 has one manure pit which is 1.2 m wide, 22.8 m long and 0.9 m deep.

*Herd description and housing prior to complete depopulation in May 2009*

Naïve sows and gilts were placed on site approximately two years prior to the described outbreak; no new animals were brought to the facility from the time of initial population in December 2007 until depopulation in May of 2009. Sows were cross-bred, specific-pathogen-free (SPF) animals [PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), and porcine parvovirus (PPV) naïve] of mixed parities. Sows and gilts were housed in buildings 2 and 3 during gestation and moved into building 5 prior to farrowing. Building 3 also contained mature boars in addition to gestating sows. Building 4 was primarily used as a nursery unit. During the observation period, there were a total of 38 breeding animals onsite (n=12 in building 2; n=13 in building 3; n=2 in building 4, n=11 in building 5).
PCV2-ASSOCIATED REPRODUCTIVE DISEASE OUTBREAK

Outbreak detection and confirmation

The details on the timeline of the PCV2 outbreak are summarized in Figure 2. The PCV2 outbreak was first noted on January 29, 2009 when a portion of weaned two week old pigs from building 4 were routinely screened and found to be positive for the presence of anti-PCV2 antibodies based on a previously described ELISA.\textsuperscript{21} Subsequently, the presence of anti-PCV2 antibodies in the samples was confirmed by an in-house IFA assay\textsuperscript{30} and PCV2 DNA was detected by using a quantitative real-time PCR.\textsuperscript{27} Serum samples were collected from all sows and a mixture of seropositive non-viremic animals, seropositive viremic animals and seronegative viremic animals were detected in buildings 2, 3 and 5. Neither anti-PCV2 antibodies nor PCV2 DNA were detected in the serum of the two gilts housed by themselves in one half of building 4. The exact timing of infection was not deduced from this data. However, based on a high proportion of sows in which PCV2 DNA was detected without detectable anti-PCV2 antibody in building 2 (66.7\% seropositive sows) and 3 (45.2\% seropositive sows), infection was apparently most recent in these building and of longer duration in building 5 (90.9\% seropositive sows).

To further characterize the PCV2 strain that infected the herd, PCV2 open reading frame 2 (ORF2) sequencing was performed on extracted DNA from two sow serum samples and one piglet serum sample using a nested PCR as previously described.\textsuperscript{25} The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, California, USA) per manufacturers’ instructions and sequenced at the Iowa State University DNA facility. Sequences were analyzed with Sequence Scanner software v1.0 (Applied Biosystems) and compared with four common PCV2a and PCV2b strains using the basic local alignment
search tool (BLAST)\textsuperscript{39} and the isolate was identified as PCV2b (100\% homology with GenBank Accession No. EU340258).

**Reproductive parameters and clinical observations in breeding animals from May 2008 to April 2009**

Farrowing data from the 38 sows between May 2008 and April 2009 was recorded by Laboratory Animal Resources (LAR) personnel. Reproductive failure was evident in one sow that farrowed a term litter composed of 13 mummies in February 2009. Microscopic evaluation of the mummified fetal tissues revealed multifocal, severe, myocardial necrosis with mineralization. Abundant PCV2 antigen was detected by immunohistochemistry (IHC) in the fetal myocardium.\textsuperscript{35} One sow was euthanized in May 2008 due to hind limb paresis associated with a vertebral abscess. No other clinical signs were reported in the breeding herd during this time period. The approximate time of PCV2 infection of the breeding herd was between December 2008 and January 2009. The PCV2 status of 10 sows at mid-gestation (approximately 57 days; February 3, 2009) and their litter characteristics (number of mummified fetuses, stillborns, and born-alive piglets) at farrowing are summarized in Table 1.

**REMOVAL OF PCV2 FROM THE FARM ENVIRONMENT**

*Facility biosecurity protocols*

Access to the facility is through a gated entrance that is locked when employees are not present. An additional perimeter fence encloses the swine facility, where a biosecurity sign is posted. To further limit the number of personal that have access, all animal buildings are
locked at all times. At entry to the facility, a change of clothes is required. The only vehicles granted access to the swine facility include a non-swine-facility-specific snow blower and a lawn mower which are used for property maintenance. All equipment that is needed in the facility is disinfected with Virkon® S (Dupont, Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut, USA) prior to entrance through the biosecurity gate. Upon access to the facility the use of foot baths are required between all animal buildings. A custom-fitted system for air filtration was installed on the outside of air inlets in each building using air filters (3M Filtrete™ Micro Particle Reduction Filter 700, 3M Co., Ames, Iowa, USA) and custom frames. Pits are emptied once a week into a nearby lagoon. The lagoon is emptied once per year in the fall by facility employees and spread onto an adjacent field. Rodent control is done by using four bait boxes per building which are filled with a brodifacoum product (Havoc® Rodenticide Bait Pack, Hacco, Inc., Randolph, Wisconsin, USA). For bird control, foam insulation (Great Stuff™ insulating foam, Dow Chemical Company, Midland, Michigan, USA) was placed into openings between the roof and walls. Specific differences in the biosecurity protocol on animal movements between buildings, personal movements between buildings, semen usage, feed delivery, equipment disinfection, visitor entry procedures, air management, manure pit management and daily chores prior to the PCV2 outbreak and following repopulation of the herd are summarized in Table 2.

**Disinfection of the premise**

**General building.** All disposable supplies were discarded including shelving units, cleaning supplies, clothing and boots and all movable equipment (i.e. facility washer and dryer) was moved outside the building. This was followed by thorough cleaning and disinfection of the
building and equipment. Specifically, floors were scrubbed with a degreaser (PRL-Grease Free®, Pharmacal Research Laboratory Inc., Naugatuck, Connecticut, USA) followed by rinsing and disinfection with a chloride compound (Clorox® Bleach, The Clorox Company, Oakland, California, USA). The surface of the washer and dryer were cleaned with general household cleaning products and exposed to natural UV sunlight. In addition, a cycle of hot water with bleach was run through the washing machine prior to replacement into the cleaned facility. New shelving units for clothing, cleaning supplies, and other supplies were placed into the office. All tools were cleaned with water and sprayed with disinfectant (Virkon® S, Dupont, Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut, USA).

Buildings 2-5: Animals were removed from the buildings 2-5 starting on May 14, 2009. Complete depopulation of the site was achieved on May 29, 2009. Steps used in the cleaning and disinfection of the animal facilities are outlined in Table 3.

Monitoring of the disinfection success of the premise
Following application of disinfection in step 4 (Table 2), 10 swabs of each building were taken in areas which would likely contain virus (pit, floor, louvers, etc.). The surface swabs (polyester-tipped swab, Fisher Scientific Inc., Pittsburgh, Pennsylvania, USA) were collected, placed into 1 ml of sterile saline (0.9% sodium chloride solution, Fisher Scientific Inc.) and stored at -80°C until tested by quantitative PCR for presence and amount of PCV2 DNA. PCV2 DNA was found on a plastic sort panel, in 2 of 10 samples taken from the office (a shelf and under a computer cabinet), the door and floor of building 5, the drain and floor of building 4, the floor and drain in building 2, and in several swabs from the pit grate and pit
floor in all buildings (2, 3, 4 and 5). The mean log_{10} genomic PCV2 DNA/ml ± std err for these positive samples was 4.48±0.14. Other locations sampled where PCV2 DNA was not detected included gating, light switches, foot baths, water nipples, pig transport carriers, the desk in the office area, the storage room floor, louvers, electrical boxes, and fan inlets.

**Repopulation of the site**

A single building (building 5) was initially repopulated with PCV2 naïve animals (negative for anti-PCV2-IgG antibodies and PCV2 DNA in serum) at the end of August 2009 (63 days following depopulation) (Figure 1). Blood was drawn monthly and tested for evidence of seroconversion to PCV2 by ELISA. Due to an increase in size of the animals and subsequently a need for more space, a portion of the animals were placed in buildings 3 and 4 in November of 2009. Animals were placed into building 2 on March 12, 2010. Neither viremia nor seroconversion to PCV2 has been detected in any of the animals during the approximately 10 months (>300 days) of operation. The first PCV2 naïve litters were born in May 2010 (Figure 1).

**ATTEMPT TO DERIVE NEGATIVE ANIMALS FROM PCV2-INFECTED SOWS DURING THE OUTBREAK**

Piglets from sows which farrowed at the beginning of April 2009 were bled at 13 days post farrow (DPF). Of these, 15 female piglets from four litters which were not PCV2 viremic at the time of sampling were weaned at 22-27 days of age and placed into a BSL-2 facility at a different site. The pigs were housed in four separate rooms by litter. All rooms had a solid concrete floor, a separate ventilation system and one nipple drinker. The pigs were fed a
balanced, pelleted, complete feed ration free of antibiotics and animal proteins other than whey (Nature’s Made, Heartland Coop) once a day. Piglets were bled monthly; serum was used to determine levels of anti-PCV2 IgG and the PCV2 viremia status of the animals by ELISA\textsuperscript{21} and PCR\textsuperscript{27}, respectively. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. Anti-PCV2 antibodies were detectable in all piglets from all litters at DPF 13. As a numeric trend toward decline of antibodies was noted between DPF 13 and DPF 204, these antibodies were assumed to be of maternal origin (Figure 3). At DPF 145, eight pigs were serologically negative; and anti-PCV2 antibodies declined to a S:P ratio below 0.2 in the remaining two animals by DPF 176 (Figure 3). PCV2 DNA was not detected in the piglets at 13 DPF; however, by 21 DPF one pig in each of three rooms had detectable amounts of PCV2 DNA and were removed from the study. By DPF 204 100% of the remaining piglets had detectable amounts of PCV2 DNA (Figure 3). Therefore, the study was terminated and pigs were euthanized.

**DISCUSSION**

The objectives of this study were to document a natural outbreak of PCV2-associated reproductive disease in a closed, high health population, to describe the procedure used to disinfect the premise before repopulation with PCV2-naïve animals, to report the outcome after repopulation, and to attempt to derive PCV2 negative animals by offsite segregation. The PCV2 outbreak in the research breeding herd was originally detected based on serological evaluation of recently weaned piglets and later confirmed by PCV2 IHC staining on mummified fetuses, detection of anti-PCV2 antibodies by IFA and ELISA and detection of PCV2 DNA by PCR on serum samples from sows. Viremia was detected in eight mid-
gestation sows (approximately 57 days) and PCV2 antigen was demonstrated by IHC on mummies from one sow. This is consistent with previous studies involving experimental-PCV2 infection of sows indicating that early infection (1-35 days of gestation) resulted in embryonic death\textsuperscript{12}, irregular returns to estrus\textsuperscript{18}, pseudopregnancy\textsuperscript{10} or small litter sizes;\textsuperscript{38} infection at mid gestation (35-70 days) resulted in mummified fetuses and abortion;\textsuperscript{12} and infection during late gestation (70-115 days) resulted in mummified fetuses\textsuperscript{9}, stillborns\textsuperscript{23}, weak-born piglets\textsuperscript{22}, delayed farrowing\textsuperscript{13}, normal litters\textsuperscript{15}, or abortion.\textsuperscript{3}

The exact timing of PCV2 infection in the research breeding herd remains undetermined. It can be assumed that initial infection occurred sometime after December 11, 2008 (last batch of naïve animals taken from the herd) and before January 27, 2009 (first detection of PCV2 in weaned pigs). Similarly, it is difficult to retrospectively determine the building into which PCV2 was first introduced. However, based on a higher proportion of sows with anti-PCV2 antibodies the introduction of PCV2 to the site is assumed to have occurred in building 5. The source strain of infection was determined to be a PCV2b isolate. Similar isolates were used commonly in a research laboratory 8 km to the North. Horizontal transmission likely occurred from either contaminated equipment or people. However, there are a large number of possible routes by which horizontal transmission may have occurred due to the design of the facility and concurrent responsibilities of personnel who visited the research herd.

Attempts to derive PCV2 negative animals from the herd following the outbreak by offsite segregation by litter were unsuccessful. In the current investigation, pigs which were non-viremic at 13 DPF were separated by litter and placed in groups of three to four animals in an
off-site BSL-2 facility. As soon as animals became PCV2 viremic, they were removed from
the study. By 204 DPF all the remaining animals were viremic. The inability to derive
negative pigs was likely due to the presence of an active PCV2 outbreak and localized
persistent PCV2 infection of the piglets with viral replication following the decline of
maternal antibodies.

Major risk factors which were identified following the outbreak included frequent movement
of animals and people between buildings with minimal biosecurity, lack of maintaining a
shower-in-shower-out facility, preparation of semen extender at an off-site facility, use of a
common tractor between the swine facility and other areas of the farm, lack of concrete paths
between buildings, and transport of feed directly into the facility. To address these issues,
specific changes were made to the facility itself and to the way in which the facility was
operated. The major changes included the enforcement of strict biosecurity protocols for
movement of people and equipment into and on the facility, the addition of concrete paths
between buildings which were put in place to reduce organic contamination of boot baths,
installation of a building for feed fumigation, and the purchase of PCV2 negative semen
(based on PCV2 PCR testing of each semen batch). Another minor change included the
addition of air filters over inlets and outlets. This change was considered minor as only one
site containing pigs (housed approximately 100 sows) is within a five mile radius of this site.
In addition, while little information exists on aerosol transmission of PCV2, in the single
study which evaluated the presence of PCV2 DNA in aerosol samples, PCV2 DNA was not
detected. The risk of PCV2 transmission from rodents or birds was also considered low and
modifications to the current rodent control protocol were not made. Previous studies have
demonstrated PCV2 replication in mice\textsuperscript{4} and a 2010 study reported that 65\% and 23.8\% of the mice and rodents from PCV2 infected swine premises were PCV2 positive, respectively.\textsuperscript{14} However, PCV2 was not detected in rodents outside of the premises.\textsuperscript{14} This information combined with the distance to the nearest swine farm makes transmission from rodents unlikely. To date no information exists to the author’s knowledge on whether indirect transmission of PCV2 can occur between avian and porcine species. However, due to lack of detection of PCV2 in species other than swine\textsuperscript{33} and the host specificity of circoviruses within avian species\textsuperscript{36}, transmission by this route seems unlikely.

Disinfection of a facility contaminated with PCV2 is an arduous task. PCV2 is known to be shed by numerous routes including nasal and oral secretions, urine and feces.\textsuperscript{34} In addition, viremia can persist in animals for extended periods. PCV2 viremia was previously reported in pigs for 140 days post infection.\textsuperscript{26} PCV2 has been shown to be transmitted both by horizontal\textsuperscript{1} and vertical routes\textsuperscript{16}. The virus is also extremely stable\textsuperscript{24,37} and many disinfectants do not completely eliminate the virus under \textit{in vitro} conditions.\textsuperscript{11,17,31} Clinical disease associated with PCV2 in breeding herds is rarely observed and typically resolves following exposure of a potentially naïve population to PCV2 between 8 and 20 weeks following the initial detection.\textsuperscript{23,32,38} Vaccination programs have been shown to be highly effective in reducing mortality associated with PCV2 infection\textsuperscript{28}; however, they do not eliminate shedding of PCV2. Based on the combination of the above factors, PCV2 naïve breeding herds are extremely rare. However, high health animals free of common viruses and bacteria are required for researchers to further advance understanding of the pathology and
epidemiology of swine pathogens. Therefore, documentation of the ability to successfully eliminate PCV2 from a farm is important.

In this study, the combination of thorough removal of organic material using a detergent, followed by exposure of equipment to natural UV light and multiple applications of a disinfectant were used. In addition, all disposable equipment was discarded and surfaces which would likely retain virus (pit covers, gating, cement, etc.) were either painted or sealed. Finally, the facility remained without animals for 63 days. As downtime was combined with thorough cleaning and disinfection, it is unknown whether downtime alone would have led to a similar elimination of PCV2 from the premise. However, the detection of PCV2 DNA within the pits following thorough cleaning and disinfection suggests that downtime alone would not be enough to prevent transmission from a contaminated building to naïve animals. After using the described decontamination protocol and enforcing a strict biosecurity protocol, the repopulated herd has remained PCV2 naïve (based on routine PCR and ELISA screening) for at least 300 days.

IMPLICATIONS

- The current study documented a natural outbreak of PCV2 in a high health research breeding herd, and similar to previous work, increased numbers of mummies were the major clinical sign.
- Several procedures used on the farm were identified as potential risk factors for transmission of PCV2 and were modified following the outbreak. The factors included frequent movement of animals and people between buildings with minimal
biosecurity, lack of maintaining a shower-in-shower-out facility, preparation of semen extension at an off-site facility, use of a common tractor between the swine facility and other areas of the site, and the lack of concrete paths between barns.

- The described procedure used for disinfection of the premise and enhanced biosecurity protocols have resulted in the maintenance of PCV2-free animals for approximately 10 months (300 days) to date.

- Attempts to derive PCV2 negative replacement animals from the herd during the outbreak by offsite segregation by litter were unsuccessful.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the many Laboratory Animal Resources personnel, especially Roger King, who carried out the cleaning and disinfection of the swine facility, and Paul Thomas and Dr. Hui-Gang Shen for assistance with laboratory and animal work.

REFERENCES


Table 1: Dam PCV2 antibody and viremia status at approximately 57 days of gestation (3-Feb-09) and characteristics of their litters at the time of farrowing where M = mummies, S = stillborns and BA = Born Alive

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</tr>
<tr>
<td>397</td>
<td>3</td>
<td>0.498</td>
<td>Positive</td>
<td>4.14</td>
<td>0</td>
</tr>
<tr>
<td>836</td>
<td>3</td>
<td>0.044</td>
<td>Negative</td>
<td>3.97</td>
<td>0</td>
</tr>
</tbody>
</table>

* Sample to positive ratio (S:P ratio)
Table 2: Outline of specific biosecurity protocols used at the site prior to the PCV2 outbreak and following repopulation of the herd.

<table>
<thead>
<tr>
<th>Task</th>
<th>Protocol prior to outbreak</th>
<th>Protocol following repopulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal movement between buildings</td>
<td>No PCV2 testing prior to movement of animals between buildings</td>
<td>PCV2 testing (PCR and serology) of animals prior to movement between buildings. Animals must be PCV2 negative</td>
</tr>
<tr>
<td>Pig transport carrier not specific to the swine facility</td>
<td>Semi-enclosed, custom built cart for pig transport used only in the swine facility</td>
<td></td>
</tr>
<tr>
<td>Non-swine-facility-specific tractor* used to transport crate</td>
<td>Non-swine-facility-specific tractor* is no longer used</td>
<td></td>
</tr>
<tr>
<td>Routine washing and disinfection of transport crate after usage</td>
<td>Routine washing and disinfection of transport crate prior to and after usage</td>
<td></td>
</tr>
<tr>
<td>Personnel movement between buildings</td>
<td>Street shoes were changed in the locker room in the general building</td>
<td>Street shoes are changed in the laundry room; separate general building designated boots are used in each room of the general building</td>
</tr>
<tr>
<td></td>
<td>Shower is required at entry to the facility</td>
<td></td>
</tr>
<tr>
<td>Between animal buildings, clothes change or showering is not required</td>
<td>Between animal buildings, clothes change and showering are required</td>
<td></td>
</tr>
<tr>
<td>No gloves were worn between office and animal buildings</td>
<td>Separate latex examination gloves worn between office and animal buildings. Gloves are disposed of prior to entry into animal building.</td>
<td></td>
</tr>
<tr>
<td>One set of boots worn between office and animal buildings and also inside animal buildings</td>
<td>Specific boots worn between office and animal buildings which are removed prior to entry</td>
<td></td>
</tr>
<tr>
<td>No designated path and no sidewalk between buildings</td>
<td>Concrete paths from building to building were installed to minimize debris entering the building</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Specific boots and separate latex examination gloves are worn in each building</td>
<td></td>
</tr>
<tr>
<td>Semen</td>
<td>On farm source</td>
<td>Commercial source</td>
</tr>
<tr>
<td>Semen was diluted on-site using extender re-constituted in an off-site laboratory</td>
<td>Diluted semen is purchased</td>
<td></td>
</tr>
<tr>
<td>Semen not tested for PCV2 prior to use</td>
<td>Semen tested by PCV2 PCR prior to entry into the facility</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: (Continued)

<table>
<thead>
<tr>
<th>Task</th>
<th>Protocol prior to outbreak</th>
<th>Protocol following repopulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed delivery</td>
<td>Feed unloaded from feed delivery truck at the general building</td>
<td>Feed truck unloaded from feed delivery truck at a gate near the main road (800 m from the general building)</td>
</tr>
<tr>
<td></td>
<td>Transported to the general building with a non-swine-facility-specific tractor*</td>
<td>Transported to the general building by facility personnel by hand following fumigation with (Virkon® S, Dupont, Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut, USA)</td>
</tr>
<tr>
<td>Equipment</td>
<td>Non-swine-facility-specific tractor* allowed in areas between buildings</td>
<td>The non-swine-facility-specific tractor* has no access to the swine facility</td>
</tr>
<tr>
<td></td>
<td></td>
<td>All equipment is re-disinfected within the general building prior to movement into the animal buildings</td>
</tr>
<tr>
<td>Entry procedure</td>
<td>Visitor sign-in book was located in room 2 of the general building</td>
<td>Visitors sign-in book directly upon entry into room 1 of the general building</td>
</tr>
<tr>
<td></td>
<td>No strict policy was in place for downtime</td>
<td>All visitors must have a minimum 72 hrs down-time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biosecurity protocol is posted on entry door into the facility</td>
</tr>
<tr>
<td>Air</td>
<td>Air filter present over air inlets</td>
<td>Air filters are present over air inlets and outlets</td>
</tr>
</tbody>
</table>

*Tractor is also used in cattle, horse and small ruminant facilities in close proximity to the swine facility.
Table 3: Stepwise cleaning and disinfection protocol used following the PCV2 outbreak.

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All equipment in all buildings including gating, crates, and pit cover plates were washed with 71.1°C water using a high-pressure (1000 psi) nozzle. Once cleaned, the equipment was removed from the building and stored outside on flatbed wagons, scaffolding, and hand carts that had constant, natural UV exposure. All water lines, gas lines, and heaters inside the buildings were removed and discarded. All fluorescent bulbs were discarded and exposed electrical parts of light fixtures were covered with tape.</td>
</tr>
<tr>
<td>2</td>
<td>Using portable lighting, the buildings, including the ceiling, light fixtures and outlets, controls, fan blades, louvers, and pits, were scrubbed by hand with brushes using a detergent (PRL-Grease Free®). Following scrubbing, the detergent was allowed 10-15 min of contact time prior to washing with a high-pressure washer as described in Step 1.</td>
</tr>
<tr>
<td>3</td>
<td>Following the initial cleaning phase, the buildings stood empty for several weeks while general repairs were made (holes in the walls were repaired, the brick was replaced where necessary). All metal pit covers were painted on both sides with a primer (DTM Bonding Primer, Sherwin Williams, Cleveland, Ohio, USA) and paint (PRO INDUSTRIAL™ Pre-catalyzed waterbased semi-gloss epoxy, Sherwin Williams, Cleveland, Ohio, USA). Approximately 216 g of an oxidizing agent (Virkon® S) was placed into the pits which were filled two thirds of the way full with water for 3 days. Following removal of the disinfectant, the concrete in the buildings was sealed (Concrete seal, product #2977, Spartan Chemical Company, Inc., Maumee, Ohio, USA). During this general repair phase, the building was disinfected three times with a chloride compound (Clorox® Bleach, The Clorox Company, Oakland, California, USA).</td>
</tr>
<tr>
<td>4</td>
<td>Disinfection of the buildings was done by applying a primary disinfectant at the manufacturer’s recommended concentration (Synergize®, Preserve International, Memphis, Tennessee, USA) using a 56.8 liter sprayer (Fimco Industries, Dakota Dunes, South Dakota, USA). The disinfectant was allowed to completely dry (2-3 days). The building was then fumigated with a second disinfectant (Virkon® S) at the manufacturers’ recommended concentration. Again, the buildings were allowed to completely dry (2-3 days).</td>
</tr>
<tr>
<td>5</td>
<td>The entire interior of the buildings including ceiling, walls, light fixtures, gating and doors was spray painted (PRO INDUSTRIAL™ Pre-catalyzed waterbased semi-gloss epoxy, Sherwin Williams, Cleveland, Ohio, USA). After the paint dried, new light bulbs, heaters, gas lines, PVC water lines, cabinets, water line hook-ups, feed barrels, garbage cans, gating and garden hoses with associated hangers were installed into the buildings. In addition, PVC boot racks were installed on the outside and inside of entrances to buildings.</td>
</tr>
<tr>
<td>6</td>
<td>The buildings were re-disinfected as described in Step 4.</td>
</tr>
</tbody>
</table>
Figure 1: Diagram of the swine research breeding facility.
Figure 2: Time-line of the PCV2 outbreak detection and repopulation of a naïve research breeding herd.

- **11-Dec-08**: Last batch of PCV2-naïve piglets was farrowed at the breeding herd facility.
- **27-Jan-09**: Weaned pigs were moved from the breeding herd facility (building 4) to a BSL-2 facility at a distant location.
- **29-Jan-09**: All sows were bled at the breeding facility. A mixture of PCV2 seropositive and viremic animals were detected in buildings 2, 3, and 5.
- **3-Feb-09**: Weaned pigs in BSL-2 facility were bled and found to be seropositive for PCV2 and PCV2 viremic. Sequencing confirmed the presence of PCV2b.
- **11-Feb-09**: One of 10 sows did not farrow on the expected date; 13 mummies were expelled.
- **29-May-09**: Complete depopulation of the breeding herd.
- **31-Jul-09**: Repopulation of building 5 is.
- **17-Nov-09**: Repopulation of half of building 3 and building 4.
- **15-Dec-09**: Repopulation of the other half of building 3.
- **12-Mar-10**: Repopulation of building 2.
- **07-May-10**: All animals in all buildings were seronegative for PCV2.
- **19-May-10**: First PCV2 negative litters.
Figure 3. Mean pig anti-PCV2 sample-to-positive (S:P) ratio (line) and percentage of PCV2-PCR positive piglets (bar) at different days post farrowing.

![Graph showing mean anti-PCV2 IgG S:P ratio and percentage of PCV2 PCR positive pigs over time.](image-url)
CHAPTER 8. General Conclusions

At the time of this dissertation, little published information exists on the diagnostic agreement between serological assays used to determine the timing of PCV2 infection. In addition, it is known that horizontal transmission of PCV2 occurs by direct contact with infected animals or their secretions; however, limited information is available on indirect horizontal transmission of PCV2.

The first objective of this dissertation was to address the question of whether commercial and in-house serological assays for PCV2 are in agreement. Because receiver operating characteristic analysis has become an increasingly valuable tool for comparison of diagnostic tests (Gardner and Greiner, 2006; Zweig and Campbell, 1993) this method was used instead of the kappa statistic which has limitations in agreement analysis (Sim and Wright, 2005). Interestingly, all four assays evaluated had high area under the curve (AUC) values indicating good discrimination between known positive and negative samples. This information alone would suggest that practitioners could choose either an in-house or commercial assay and receive similar results. However, assays which had similar, high AUC values had differences in sensitivity and specificity using laboratory specific cut-offs. This implies that for accurate depiction of trends in the sero-prevalence of PCV2 in herds, samples should be consistently run on the same serological assay. This is due to the variation noted in the sensitivity of the assays when laboratory specific cut-offs were applied to the data set.

The analysis using laboratory specific cut-offs also indicated that (1) the usefulness of both the commercial and the in-house assay is limited early in the infectious process due to lower sensitivity; (2) later in the infectious process, the in-house assay had a higher sensitivity and
specificity in comparison to commercial assays; (3) early in the infectious process, IFA assays may provide greater utility in comparison to ELISAs; and (4) the limited number of samples tested in this trial may have been an important factor for the differences in optimized and established cut-offs; therefore, further studies with larger sample sizes are necessary to determine an appropriate cut-off for these assays. A secondary finding was that currently offered assays are detecting both anti-PCV2a and anti-PCV2b antibodies following both natural infection and vaccination. As there has been a global shift of PCV2 genotypes from PCV2a to PCV2b (Dupont et al., 2008), it is important for veterinary diagnosticians and practitioners to be confident current assays are detecting both genotypes.

The second objective of this dissertation was to gain insight into several aspects of horizontal transmission. Vital to this objective is knowledge of characteristics of the virus, including but not limited to routes of shedding, amount of virus present in various secretions and excretions, stability, and efficacy of disinfection protocols. Previous information has documented that PCV2 is shed by numerous routes including nasal and oral secretions, urine and feces (Segalés et al., 2005b); however, limited or no information is available on the quantity and infectious nature of the detected PCV2 in these samples. In addition, it is known that viremia can persist in animals for extended periods; PCV2 viremia was detected in animals at 140 days post inoculation (DPI) (Opriessnig et al., 2010). PCV2 is also extremely stable (O'Dea et al., 2008; Welch et al., 2006) and many disinfectants do not completely eliminate the virus under in vitro conditions (Kim et al., 2009; Royer et al., 2001). The combination of these viral characteristics and the high worldwide seroprevalence (Ramamoorthy and Meng, 2009) provide evidence that horizontal transmission is likely an important factor in maintenance of the virus in swine populations. To date, the majority of
published literature addresses vertical transmission or horizontal transmission through direct contact. Little information is available on the role of indirect contact in the transmission of PCV2. Specific questions that have not been previously addressed which were evaluated in this work included the following: (1) how long is PCV2 DNA present in various secretions and excretions, (2) is the PCV2 DNA detected in secretions and excretions infectious to naïve animals, (3) does routine disinfection of commercial transport trailers prevent transmission of PCV2 to naïve animals, and (4) can PCV2 remain infectious during the spray drying process and be spread to naïve animals via spray-dried plasma.

To answer the question of how long PCV2 DNA can be detected in various secretions from PCV2-infected animals and whether the detected PCV2 DNA was infectious, a cohort and bioassay study were designed. Specifically, naturally PCV2-infected pigs and PCV2-naïve pigs experimentally inoculated with PCV2 at 21 days of age were routinely sampled (fecal, oral and nasal swabs and serum samples) until 209 days post-farrowing (DPF; natural infection) or until 69 days post-inoculation (DPI; experimental infection). The results of this study documented the longest period of PCV2 viremia under controlled conditions (181 day observation period). It was also demonstrated that in both experimental and natural PCV2 infections, PCV2 is shed in high amounts in nasal, oral and fecal excretions for extended periods of time. In addition, naïve animals were inoculated by various methods with different samples (oral fluid, nasal secretions, feces or a contaminated needle) taken from the experimentally infected animals at the time of expected peak shedding. This study confirms that naïve animals can be infected with PCV2 present in nasal secretions, oral secretions and fecal material via the intraperitoneal route, with PCV2 present in feces via the oral route, with PCV2 present in nasal secretions via the intranasal route and potentially through
contaminated needles. However, future studies in which replicates of naïve animals are inoculated with serially diluted concentrations of PCV2 DNA are needed to identify the infectious dose for each route of exposure.

The question of whether routine disinfection of commercial transport trailers prevents transmission of PCV2 to naïve animals is of concern to practitioners and producers as large numbers of animals are transported between facilities on a daily basis. Previous work has shown oxidizing agents, aldehyde compounds and quaternary ammonium compounds have efficacy against PCV2 under in vitro conditions (Kim et al., 2009; Royer et al., 2001) (11, 20). While in vitro efficacy data is useful, field efficacy of a disinfectant depends on the type of surface and the presence of organic material among other variables (Amass, 2004). To accurately simulate field conditions, 1:61 scale model trailers were designed and manufactured by a company specializing in standard and custom aluminum livestock transportation equipment with the exact materials used during commercial production. Trailers were manufactured purposely to contain areas where fecal material would likely accumulate and sampling of the trailers was concentrated on these areas to accurately determine viral concentration within the trailer following each step in the protocol. The results of this study indicate that following the disinfection step, between 1.5 and 5.2 log$_{10}$ PCV2 genomic copies/ml were identified in the trailers and only one disinfection protocol (DISF 4; an application of two disinfectants in tandem, the first being an oxidizing agent (Virkon®S, Pharmacal Research Laboratories Inc., Naugatuck, CT) followed by a chloride compound (Clorox® Bleach, The Clorox Company, Oakland, California, USA) significantly reduced the log$_{10}$ PCV2 genomic copies/ml compared to the log$_{10}$ PCV2 genomic copies/ml present in the post-wash step. Interestingly, although the trailers were contaminated with a
consistent, high amount of virus ($10.8 \log_{10}$ PCV2 genomic copies/ml) and PCV2 DNA was detected in trailers following disinfection, transmission to naïve animals within a two hour period did not occur. As $1.45 \pm 0.6 \log_{10}$ PCV2 genomic copies remained in the trailer following the most efficacious disinfectant protocol, it is possible that longer exposure periods could have resulted in transmission of PCV2 to naïve animals. In addition, it would be of benefit to repeat the study to increase the probability of detecting infection. Also of interest would be a comparison between the infectious dose of PCV2 needed to initiate infection using this model in comparison to other routes including intraperitoneal, oral, etc.

The last potential mechanism for horizontal transmission of PCV2 evaluated in this dissertation is horizontal spread through spray-dried plasma (SDP) products. Several previous studies have reported the lack of viable pseudorabies virus (Polo et al., 2005), porcine reproductive and respiratory syndrome virus (Polo et al., 2005), and swine vesicular disease virus (SVDV) (Pujols et al., 2007) within SDP. In contrast to PRRSV, PRV and even other stable, non-enveloped viruses such as SVDV (Turner and Williams, 1999), PCV2 has been shown to be extremely resistant maintaining viability at temperatures of 60°C for 24 hrs and 75°C for 15 min (O'Dea et al., 2008; Welch et al., 2006). In addition, although inlet temperatures of 240°C can be achieved during this process, the time in which the product is exposed to this temperature is short. This design was made to enable efficient drying of plasma products without the destruction of various proteins including antibodies to PCV2, *Mycoplasma* sp., PRRSV, TGE and SIV (Borg et al., 2002). Last, a previous study detected PCV2 DNA in commercially SDP but failed to determine whether it was infectious or not in PCV2 naïve animals (Pujols et al., 2008).
It was therefore, the objective of this portion of the dissertation to use a bench-top commercial spray-dryer according to the manufacturer’s recommendations to spray-dry experimentally infected plasma. Following generation of the SDP, it was intraperitoneally inoculated into PCV2-naïve animals to determine the ability of PCV2 to withstand the process. Reasons for using this method in contrast to the previous study by Pujols et al., included: (1) elimination of the question of whether maternal antibodies affect infection following consumption of SDP containing PCV2 DNA and (2) use of the most sensitive method available (swine bioassay) to detect the presence of PCV2. The results of this study provided direct evidence that porcine SDP collected from a PCV2b-experimentally infected pig is infectious to naïve animals through the intraperitoneal and oral gavage route. This combined with the knowledge that PCV2 DNA is present in commercially produced SDP (Pujols et al., 2008) indicates that SDP sourced from pigs could represent a biosecurity risk for the swine industry. However, due to the large number of studies which have cited favorable production parameters following the incorporation of SDP into nursery diets, further studies are necessary to validate the infectivity of PCV2 DNA in PCV2-naïve pigs through incorporation into the diet (i.e. a realistic exposure model) and to determine the infectious dose of PCV2 via this route of exposure.

While animal work was ongoing for the data discussed above, a unique opportunity arose to implement the knowledge gained from the previous work in the field. Specifically, the swine herd supplying PCV2 naive animals for these studies underwent a natural PCV2 outbreak and the decision was made to roll the herd over in order to allow future research to continue. Therefore, it was the final objective of this dissertation to document a natural outbreak of PCV2 in a specific disease free population, to describe the procedure used to
disinfect the premise, and to document the attempt to derive PCV2 negative animals for use as replacement stock in the facility. The PCV2 outbreak was originally detected based on serological evaluation of recently weaned piglets and later confirmed by immunohistochemistry staining on mummified fetuses, IFA, PCR and sequencing of samples from sows and a piglet. Clinical signs were absent in sows, with increased numbers of mummified fetuses being the primary clinical sign. This is similar to previous case reports in which outbreaks were characterized by an increased percentage of mummies and stillborns within the population with minimal clinical signs noted in the dams (O'Connor et al., 2001; West et al., 1999). The exact timing of infection was not determined due to the variation in serological and viremia response in the sows. However, sequencing of the ORF2 region of the PCV2 recovered from both a sow and piglet during the outbreak had 100% nucleotide homology with NC-16845 isolate (GenBank#EU340258) which was used commonly in a nearby (8km) research laboratory for experimental infection of naïve animals. The premise was depopulated and cleaned by thorough removal of organic material using a detergent, exposure of equipment to natural UV light followed by multiple applications of a disinfectant. All disposable equipment was discarded and surfaces which would likely retain virus (pit covers, gating, cement, etc.) were either painted or sealed. In addition, a strict biosecurity protocol was put into place to minimize the risk of horizontal transmission. Using the described decontamination protocol and enforcing a strict biosecurity protocol, the site was re-stocked with PCV2-naïve pigs 63 days later and the population has remained PCV2 naive (based on PCR and ELISA screening) to date (>300 days). However, an attempt to derive PCV2 negative replacement animals from non-viremic piglets weaned during the outbreak was not successful. While, the majority of herds worldwide are seropositive for
PCV2, documentation of the disinfection process used in this study is important to producers and researchers and likely can be extrapolated to herd roll-overs following contamination with other viral pathogens.

In summary, the research described in this dissertation provides significant contributions to the knowledge of horizontal transmission of PCV2 specifically relating to the role of transmission of PCV2 through contact with contaminated secretions, transport trailers, and spray-dried plasma. In addition, this work provides information on the diagnostic agreement among serological assays and documents the roll-over of a specific pathogen free herd following a natural PCV2 infection.

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ACKNOWLEDGEMENT

Thanks to my committee members for their mentoring, guidance and encouragement throughout this project.

A special thanks to Drs. Darin Madson, Phil Gauger, Kent Schwartz, Rodger Main and Dianna Jordan. Without your support and encouragement, I probably wouldn’t have seen this project through.

Thanks to Shayleen Harrison, Paul Thomas, Matt Umphress, Joe Bender and a number of other veterinary students for all their help in carrying out the studies.

Thanks to my husband for patiently waiting for his “return on investment” – one decade in post-secondary education is enough for now… To both my husband and daughter, thanks for your love and support.

Last but definitely not least, a very special thanks to my parents who always inspired me to work hard, be the best person I can and to try to be as good a parent as they have been. Without your support, encouragement, love, phone calls, help babysitting and everything else you have done for me I would not be the person I am today.