UNIVERSITY OF MINNESOTA

PREVALENCE OF PORCINE CIRCOVIRUS TYPE 2 (PCV2) IN THE U.S. NATIONAL SWINE HERD

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ABBREVIATIONS

Capsid	Cap
Enzyme-linked Immunosorbent Assay	ELISA
Indirect Immunofluorescence	IIF
Immunoperoxidase Monolayer Assay	IPMA
Keyhole limpet hemocyanin	KLH
Number	N
National Animal Health Monitoring System	NAHMS
Optical Density	OD
Open Reading Frame	ORF
Porcine Circovirus 2	PCV2
Porcine Circovirus Associated Disease	PCVAD
Porcine reproductive and respiratory syndrome virus	PRRSV
Porcine parvovirus	PPV
Postweaning Multisystemic Wasting Syndrome	PWMS
Replicase	Rep
United States Department of Agriculture	USDA

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Porcine circovirus type 2-associated disease (PCVAD) causes significant economic damage in the swine industry. Although other environmental and pathogenic triggers can influence the occurrence of PCVAD, PCV2 is the key etiological pathogen for this disease (1). The scope of PCV2 incidence in the US national swine herd was evaluated in 2006 by the National Animal Health Monitoring System (NAHMS), initiated by USDA. At that time, which was before vaccines were available in the U.S., over 82% of sera from 185 farms were DNA positive and more than 80% were also positive for anti-PCV2 antibodies, which indicate that PCV2 was widespread and a persistent infection in pigs (2). Commercial vaccines have been widely used in US since then. Another NAHMS assessment of swine health was carried out in 2012. This thesis is focused on the immunologic evaluation of PCV2 status and a determination of both vaccination and infection status.

Virus

History: Porcine circovirus (PCV) was first identified as a contaminant in the porcine PK-15 cell line in 1974 (3). The virus was shown to have a diameter of 17 nm, and to contain a covalently closed single-stranded DNA (3). The new virus was designated as Porcine circovirus type 1 (PCV1) and was found to be nonpathogenic to swine (4), although serological surveys revealed there was a high prevalence of PCV1 antibodies in the swine population (5). Later, a variant strain of PCV was associated with a newly emerged clinical symptom that become known as post-weaning multisystemic wasting syndrome (PMWS) (6). Subsequently, nucleotide sequence analysis of the PCV associated with cases of wasting syndromes from North America and Europe revealed it has an 68% homology with the previously known PCV derived from PK-15 cells, indicating these two strains appeared to be closely related but still distinct from each other (7, 8). This pathogenic PMWS-associated PCV was then referred to as PCV2 to distinguish it from the original PK-15 cell culture isolate, PCV1 (7). A retrospective investigation showed that PCV2 was present in pigs in 1962 in Northern Germany (9), but not until the late 1990s outbreaks reported simultaneously in various countries (10, 11). Since then, PCV2-associated disease has become one of the most economically significant swine diseases (12).

Classification: PCV2 is a member of the genus *Circovirus* in the family *Circoviridae*, which is a non-enveloped virus, consisting of a single-stranded, circular DNA genome (3, 13).

Composition: A total number of 1767-1768 nucleotides comprise the PCV2 genome, of which 11 putative open reading frames (ORFs) are predicted (8), but only 4 of which have been characterized.

The largest open reading frame of PCV2 encodes a replicase (Rep) of 312 amino acids and its splicing variant Rep'(14)(15). Rep protein alone cannot promote replication; Rep and Rep' together comprise the functional replication initiator factor of PCV2 (16). ORF2 of PCV2 encodes the major structure protein, which builds the virus capsid (17). The capsid protein has also been identified as the major immunogenic and protective protein of PCV2 (18). A 315-bp gene encodes a novel viral protein (ORF3 protein), which may be involved in PCV2-induced apoptosis in cultured cells (19), but it has been suggested that the pathogenicity of PCV2 is either not determined or not solely determined by the ORF3 protein (20). A fourth viral protein, ORF4, is newly discovered. ORF4 is not essential for PCV2 replication but plays a role in suppressing caspase activity and regulating CD4 (+) and CD8 (+) T lymphocytes during PCV2 infection (21). Although many proteins in porcine cells were identified to interact with ORF1 (Rep/Rep'), ORF2 (Cap), and ORF3 (22-24), a specific molecule or pathway that triggers PMWS or other PCVAD has not been fully described (25).

In 2012, a new agent named P1 was detected by PCR during a diagnostic study of PCV2 in Hebei and Jiangsu, China (26). The sequenced genome of this porcine circovirus-like agent is composed of a 648-nucleotide circular DNA, which includes three predicted ORFs (27). Since P1 does not share the replication origin with PCV2, it may be a result

of recombination of PCV2 of other organisms rather than rearranged (28). P1 may be a new member of the family *Circoviridae* (27) or possibly a new porcine circovirus (28).

Genetics: PCV1 and PCV2 have similar genome organization and nucleotide homology of approximately 68% (8). At first, a close phylogenetic relationship among global PCV2 isolates was shown based on nucleotide sequence similarities greater than 93%. The methodology of distinguishing PCV2 genotypes depends on the proportion of nucleotides sites at which two sequences being compared are different (p distance). This value is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared. The sequences with nucleotide diversity cut-off of 3.5% could be divided into two major groups PCV2a and PCV2b (29). A third genotype (PCV2c) was reported from Denmark in the 1980s. Two additional genotypes, PCV2d and PCV2e, were suggested (30), however, subsequent analysis of the sequence data could not support the new classification (31).

PCV2a was the most clinically prevalent genotype from 1996 to the early 2000s (32). Different countries (Canada, Denmark, Spain, Sweden, Switzerland and USA) have described a shift on genotype prevalence from PCV2a to PCV2b, coinciding with the advent of the most severe outbreaks of PCVAD (1, 32-37).

Pigs are also co-infected with these two genotypes. Both PCV2a and PCV2b were presented in 25% of the clinical samples in a US study in 2008 (38), while in 2011, this co-existence rate was 32.2% in another study from China (39). A recent study in experimentally infected pigs indicated that single infection with either PCV2a or PCV2b

was not associated with clinical disease but concurrent infection of pigs with both genotypes could result in PCVAD (40).

Geographical distribution: It appears that Europe and North America have been the major areas of PCV2 spreading all over the world and, moreover, that PCV2b isolates were apparently introduced in North America from Europe (1, 97). High PCV2 seroprevalence of >92% has also been reported in Mexico City backyard pigs (98).

In Asian countries, PCV2 also has a deep impact on the swine industry. In Korea, PCVAD is a leading cause for economic concern within the pork industry since the first outbreak in 1999; however, only PCV2b strains have been isolated from pigs with PMWS since 2005 (93). In China, the genetic variation of Chinese PCV2 strains is similar to North American and European strains. The first commercialized vaccine, CircoFlex®, was registered in 2009 to control PCV2 infection and PCVAD. Several killed vaccines derived from whole virus of some Chinese PCV2 strains were subsequently designed and also registered by Chinese local enterprises. These vaccines have been applied extensively in controlling PCV2 infection and the manifestations of PMWS and other PCVAD (99). From 2011 to 2012, PCV2b was the predominant genotype circulating in southern China (108).

Detection

To detect the nucleic acids of PCV2, *in situ* hybridization (ISH) and several kinds of PCR have been developed. *Multiplex PCR has been used to detect* more than one target

sequence (PCV1/PCV2, PCV2/PPV, PCV2/pseudorabies virus/PRV) in a single PCR reaction (41-44). Nested PCR can increase the ability to detect very small amounts of the target sequence (43). Multiplex-nested PCR assay is designed to detect very small amounts of several target sequences combining multiplex and nested PCR (45, 46). The amount of PCV2 nucleic acids in serum and tissues has been demonstrated to be predictive of the clinical manifestations, so that quantitative real-time PCR assays have been developed to determine the amount of PCV2 genomic copy numbers in the serum or tissues (47, 48). ISH for PCV2 allows localization and quantification of PCV2 but the method in general is time consuming and highly expensive (46, 49-51).

In order to study PCV2 viruses or viral antigens, immunohistochemistry (IHC), electron microscopy (EM), virus isolation (VI), and antigen-capture ELISA were developed. Virus isolation of PCV2 has been developed in the PK-15 cell line, in which PCV1 was discovered (3). However, while VR1BL cells were shown to have higher viral titers than PK-15 cells (52). IHC uses polyclonal antibodies to detect PCV2 antigens in formalin-fixed, paraffin-embedded tissue sections (49, 53). EM is used to demonstrate circovirus-like particles directly within a cell and to study the virus structure and size (54), but it not a routine diagnostic method because it is time consuming and expensive. Antigen-capture ELISA on tissue homogenates has been described and the results were found to be comparable to quantitative virus isolation and IHC (55).

There are several ways to detect the anti-PCV2 antibodies, such as indirect immunoperoxidase monolayer assay (IPMA), indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and seroneutralization (SN) assay. The

SN assay has been used to detect the presence of neutralizing antibodies that have the ability to prevent virus from attaching to and/or infecting cells (56). IFA detects the ability of the antibodies in the serum to bind to a fixed monolayer of PCV2-infected cells (6, 41, 57, 58). Recently, a quantitative immunofluorescence assay (QIFA) method was established for sero-diagnosis of PCV2 using Vero cells expressing capsid protein of PCV2 (59). The IPMA is similar to the IFA with the except that the antibodies are peroxidase-conjugated anti-swine IgG (60, 61).

An ideal diagnostic method must incorporate several features including accuracy, rapid testing, low cost, especially when applied to large-scale surveys. In this way, ELISA is relatively simple and suitable for screening a large number of sera, although quantitative analyses are still limited, considering the serum neutralization assay as a gold standard (59).

A competitive ELISA was designed to test serum for anti-PCV2 antibodies. In that assay a cell culture isolate of porcine circovirus type 2 was chosen as antigen and a PCV2-specific monoclonal antibody as the competing reagent (62). Cap-based ELISA (63-66) achieved 88.5% specificity and 89.4% sensitivity for detection of PCV2 antibody in field sera (65). Two indirect ELISA were developed based on recombinant PCV2 Cap (rCap) and Rep/Rep′ (rRep) proteins expressed in baculovirus and produced in *Trichoplusia ni* insect larvae, which is the first report that rRep ELISA as a new tool to serologically distinguish between vaccinated and non-vaccinated animals, at least for those commercial vaccines based on the Cap PCV2 protein or inactivated virus (67).

Disease

The first report of disease related to PCV2 was postweaning multisystemic wasting syndrome (PMWS), which was initially described in 1991 in western Canada as a sporadic disease, characterized by emaciation, jaundice, enteritis and dyspnea (91). The histopathology of the lymphoid tissues of those affected pigs showed a severe lymphoid depletion. Many other countries have subsequently reported similar disorders. PMWS is a disease of growing pigs with low morbidity but high case mortality, but a causal relationship between PMWS and PCV2 had not been elucidated at that time (91).

PMWS is the most common form of PCVAD and is characterized by wasting, decreased weight gain, dyspnea, jaundice and enlarged lymph nodes, as well as degeneration, necrosis of hepatocytes, multifocal lymphohistiocytic pneumonia, lymphocytic depletion and multinucleated giant cell formation (92, 93). The American Association of Swine Veterinarians (AASV) created the concept of porcine circovirus associated-disease (PCVAD) in March 2006, in which a connection with PCV2 was involved (12), while in Europe, the name of PCVD was used instead (90). Based on the case definition posted by AASV in Oct 2006, PCVAD can be subclinical or contain one or more clinical manifestations such as multisystemic disease with weight loss, high mortality, respiratory signs, porcine dermatitis and nephropathy syndrome (PDNS), enteric signs including diarrhea, and reproductive disorders individually or in combination in a herd or group of pigs (12).

PCVAD is a multifactorial disease. Although PCV2 was experimentally examined as the etiological agent (94), infection with PCV2 alone does not generally result in clinical

disease. Other factors such as variation of virus and host, co-infections and immune modulation are considered crucial for development of overt clinical manifestations of PCVAD (12). Mutations in amino acid may lead to significant attenuation of the virus, but there is no consistent pattern between PCV2 isolates from affected and unaffected animals. Pigs of all breeds seem to be equally susceptible to PCV2 infection. Different types of adaptive immune response against PCV2 seem related to the level of virus replication and clinical manifestation. For management practices, stress and/or other potential triggering factors that induce immune stimulation is important in triggering PCVAD (95, 96). Besides housing, vaccination schedules, biosecurity, hygiene and husbandry practices can modulate the expression of PCVAD. Concomitant viral and bacterial infections, such as Porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV) and *Mycoplasma hyopneumoniae,* with PCV2 can increase the incidence of PCVAD (29)(95).

Pathogenesis

PCV2 can be transmitted horizontally and vertically. The oronasal route is considered the most likely and frequent route of PCV2 transmission, but transmission can also occur through direct contact via fecal and urinary routes (87, 88). Breeding animals can also be infected with PCV2 via semen from infected boars (89).

The pathogenesis of PCV2 infection and major cell types that support PCV2 replication are still under investigation. After PCV2 infects a cell, the single-stranded DNA (ssDNA)

is converted to a double-stranded DNA (dsDNA) intermediate known as the replicative form (RF). Under commercial farm conditions, the majority of pigs seroconvert to PCV2 between 2 and 4 months of age (90).

The VR1BL cell line was proved to be superior to PK-15 cells for the study of PCV2-host cell interactions and molecular pathogenesis because it was highly permissible to PCV2 infection, induced cellular destruction, and sensitivity to viral load compared to PK-15 cell line. (52)

Control and Prevention

Before vaccination was introduced, the control of PCVAD was limited mainly to improve management strategies and controlling co-infections. Commercial PCV2 vaccines were initially developed to control PMWS, but they are now also used against other PCVAD (68).

Several kinds of vaccines against PCV2 have been designed and tested under experimental conditions, such as killed PCV2 vaccine, ORF2 subunit vaccine, DNA vaccine, pseudorabies virus expressing ORF1-ORF2 vector vaccine, adeno-ORF2 expressing vector vaccine, live or inactivated attenuated chimeric PCV2-1 vaccine (18).

In an evaluation study of PCV2 subunit vaccine, ORF2-encoded capsid protein constitutes the major immunogen to induce protection of piglets against a PCV2 challenge, while ORF1-encoded replication protein was weakly immunogenic (18).

ORF2 was chosen because it contains the main neutralizing epitope and thus has potential to induce a protective immune response (64, 69).

There are several commercial vaccines in the market, three of them are subunit vaccines based on the PCV2 capsid protein expressed in baculovirus, including Ingelvac CircoFLEX® (Boehringer Ingelheim), Circumvent® PCV M (Merck Animal Health, Inc) and Porcilis® PCV (MSD Animal Health). The Circovac® vaccine (Merial) is an inactivated PCV2 vaccine. The Fostera™ PCV vaccine (Zoetis) is an inactivated chimeric PCV1–2 vaccine, redesigned from Suvaxyn® PCV2 One Dose (70)(71, 72). Commercially PCV2 vaccines are different in their antigen, adjuvant types, recommended animals (sow or piglet or both) and dosage (one or two doses). All commercial PCV2 vaccines are based on the PCV2a genotype. Nevertheless, PCV2a-based vaccines are able to control PCV2b infection in pigs through cross-protection (73). However, a recent study showed that a PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection (74).

Co-existence of PCV2a and PCV2b may result in enhancing of PCV2 replication. A study showed an obvious vaccination failure in 30 10-week old pigs, among which only 50% developed a detectable immune response, putatively indicating a poor vaccine efficacy (70).

Current commercial vaccines have been considered highly effective to inducing protective immunity against PCVAD in the field (75-80) and under experimental conditions (73, 81, 82). Clear parameters for experimental vaccine evaluation are not

sufficient due to lack of consistent, precise animal models for PCVAD. Presence of clinical signs, presence of microscopic lesions, presence of PCV2 in lesions, and viral load in serum are the most common parameters used for evaluating a PCV2 vaccine (48, 83), while differences in growth performance in some field trials by different PCV2 vaccines may also be due to different co-infection, field management, feeding quality and production system conditions.

The efficacy of commercial PCV2 vaccines has not only been tested in PCV2 naïve pigs (84) and PCV2 negative pigs (73, 85) in experimental condition, but also in field conditions. In a field study, 1542 serum samples were collected from a farm suffering from porcine respiratory disease complex (PRDC). PCV2 vaccination decreased the PCV2 viral load by 55% to 83% and the mean duration of viremia by 50% ($P<0.0001$) (76). In another study in a herd with a history of PCVAD, PCV2 vaccination reduced the mortality by 50% and increased the daily gain in the finishing period (79). In a small controlled experimental trial, after challenging with different PCV2 isolates of different genotypes and geographic origin, vaccinated pigs had significantly reduced fecal and nasal shedding of PCV2 (73). In another controlled experimental study, PCV2 vaccination reduced PCV2 associated lesions and viremia in pigs concurrently infected with PCV2 and PRRSV (84).

PCV2 viremia has not been detected in dams of PCV2 vaccinated pregnant animals, and serum neutralizing antibodies and colostral anti-PCV2 antibodies have also been induced. However, PCV2 DNA was detected in colostral samples and individual fetuses following

oronasal PCV2 challenge which means vertical transmission of PCV2 can occur in PCV2-vaccinated dams (86).

Future Study

There are still many questions to be answered about PCV2. PCV2 immunology is a great challenge area. The nature of the factors that trigger the development of PCVAD is largely unknown. Only some individual pigs exhibit clinical signs, while others show no clinical signs and they maintain an apparently good performance. Individual expression of the disease is a combined result of the virus, the host, the infection timing, and management, but the mechanism of each factor is still under investigation. The appearance of PCVAD in different countries around the world at almost the same time is also a mystery, as well as the genotype shift from PCV2a to PCV2b around the world at the same time.

Hypothesis

I hypothesized that analysis of anti-capsid and anti-replicate antibodies would enable assessment of PCV2 exposure in the United States swine herd. In this study, 2989 serum samples from 202 farms in the NAHMS 2012 survey were tested serologically using both cap-specific and rep-specific indirect ELISA. Since all the pigs vaccinated during the 5 years received commercial vaccines based on the capsid protein, and replicase protein is

produced only by replicating virus, the comparison of the two ELISA results is expected to give us information about infection and vaccination status. These serological results are also compared with data from 5 years ago to provide us a historical trend of PCV2 infection nationwide.

Chapter 2

Materials and Methods

Indirect ELISA

Serum samples

A total number of 5,730 serum samples (30 samples from each farm of 205 farms, from 13 states) were collected as part of the USDA (United States Department of Agriculture) National Animal Health Monitoring System's (NAHMS) swine 2012 study, which focused on operations with 100 or more pigs. Approximately 15 serum samples per farm, for a total of 2989 samples from 202 farms, were taken for serological detection. Blood was collected from the cranial vena cava or jugular vein and allowed to sit vertically at 20-24°C for a minimum of 30 min. Samples were cooled to 4°C and shipped on ice within 24h to the USDA National Veterinary Services Laboratories (NVSL) in Ames, IA. Sera were drawn off, aliquoted, and frozen at -20°C until use (100).

Preparation of cap and rep antigen

Proteins were made by lab members; PCV2 capsid gene fragments is 549 bp, lacking the

amino terminal 39 and carboxyl terminal 10 amino acids, were amplified with BamH1 and Xho1 restriction enzyme sites. PCV2 cap-specific primers were 5′ -CGCGGATCCATGAAAAATGGCATCTTCAACACCCGCCT-3′ and 5′ -CCGCTCGAGTTCTCTGAATTGTACATACATGGT-3′ ; PCV2 replicate gene fragments is 963bp, were amplified with BamH2 and Xho1 restriction enzyme sites. rep-specific primers were 5'-CGC GGA TCC ATG CCC AGC AAG AAG AAT G-3' and 5'-CCG CTC GAG GTA ATT TAT TTC ATA TGG A-3' . PCR products were gel purified, digested with BamHI and XhoI (New England Biolabs, MA), and cloned into a modified pET24b vector (Novagen, Madison, WI) for expression in BL-21 (DE3)-RP cells (Stratagene, CA, USA). Gel picture see **APPENDIX1**.

Choose of non-specific background, positive and negative control

The nonspecific background control was keyhole limpet hemocyanin(KLH). Positive and negative controls were chosen from previous PCV2 studies **(APPENDIX2)**.

Indirect ELISA procedure

Plates were coated with 100ng/well of test antigens (cap/ rep/ KLH) in carbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and incubated at 4 °C overnight. Plates were then washed three times with phosphate-buffered saline with 0.05% Tween 20 (PBST,

pH7.4) at room temperature using an ELISA plate washer. Wells were filled at the end of the third wash and allowed to stand for 5 min at room temperature. The plates were then washed three more times and tapped on paper towels to remove residual wash buffer. Wells were blocked with 300 µl of 5% nonfat dry milk (NFDM), pH 9.6, and the plates were incubated at room temperature for two hours. Plates were washed and 100µl of test sera, diluted 1:50 in PBST containing 5% NFDM, pH 7.4, in duplicate for one hour. Plates were washed, and 100µl of peroxidase labeled goat anti-swine IgG (H+L) (KPL, Bethyl Labs), diluted 1:100,000 in PBST with 5% NFDM, pH 7.4, was added to each well at room temperature for one hour. The plates were washed four times and color was developed with TMB peroxidase substrate and H_2O_2 (KPL) for 15 min at room temperature. Reactions were stopped with 1 M phosphoric acid and plates were read at 450nm in a ThermoMax microplate reader. Positive results were taken as absorbance values >0.1.

Assay repeatability

Both “intra-assay” and “inter-assay” repeatability was controlled in our indirect ELISA methods. Intra-assay repeatability was measured by running the same samples and controls on duplicates in the same plate. Inter-assay repeatability was measured by involvement of positive and negative serum standards on every plate (100).

Statistical methods

Statistical tests were performed using Prism 6 for Mac OSX (GraphPad Software) and R 3.0.2 for Mac OS 10.6. Correlations were calculated by Pearson's correlation.

Chapter 3

Results

Positive and negative controls

Positive and negative controls were chosen from previous PCV2 study. With cut off of 0.4, three serums above 0.4 were chosen to pool as the positive control. One below 0.4 was chosen as the negative control **(APPENDIX 2)**.

Determination of non-specific background of ELISA

Keyhole limpet hemocyanin (KLH) is a large, multisubunit, oxygen carrying, metalloprotein that is found in the hemolymph of the giant keyhole limpet, *Megathura crenulata*. KLH is a potent immunogenic high-molecular-weight protein, is used extensively as a carrier protein in the production of antibodies for research, biotechnology and therapeutic applications. I used KLH as our non-specific background. To determine the non-specific background, I tested 48 serums with both KLH and blank wells **(Figure1)**. The average OD value with KLH is about 0.12. The average OD value with blank wells is about 0.08.

Selection of primary antibodies and secondary antibody dilutions

In order to assess the best distinguishable ELISA results for both Cap and Rep protein to determine the serological level of serum, different dilution series of serum antibodies and secondary antibodies were measured. I used 1:50, 1:100 and 1:1000 as the dilution candidates for primary antibody, while 1:50,000, 1:75,000 and 1:100,000 as the dilution candidates for secondary antibody. Two serum positive samples (Yeske 4 and positive control) and two serum negative samples (B8-267 and B8-275) were tested for both cap and rep in this experiment. **Figure 2 A, B and C** shows when the secondary antibody dilution is 1:50,000, 1:75,000 and 1:100,000, the OD values of those samples in primary antibody dilution of 1:50, 1:100 and 1:1000. From these three graphs we can conclude that, as the dilution of primary antibody gets higher, the difference of the OD values becomes less. For the sake of getting higher recognition level of the sample OD value, I choose 1:50 as my sample serum dilution. Although the secondary antibody dilution of 1:50,000 has the highest recognition level of the OD values, the OD values of negative samples are almost 0.1, not low enough to represent my negative results since my non-specific background is 0.05. Considered of all the influential factors, I decided to use 1:50 as my primary antibody dilution, while 1:100,000 as my secondary antibody dilution.

Correlation analysis of ELISA assays from NAHMS swine 2006 and NAHMS swine 2012

Because I would compare the PCV2 infectious level of NAHMS swine 2012 with NAHMS swine 2006, the measurement of the consistency of the two ELISA assays is required. The new ELISA assay was tested on serum samples from the NAHMS swine 2006. 100 serum samples, which were tested seropositively in NAHMS 2006 ELISA assay, were chosen to be detected OD values with our new NAHMS 2012 ELISA assay. I then compared the OD values of those samples from NAHMS 2006 with NAHMS 2012 assays, and linear correlation of data from these two assays was presented. The coefficiency of the two sets of data is 0.63 ($p<0.0001$), which is high enough to certificate that these two ELISA assays are quite consistent according to the different OD values of the same sample set.

Sample amount for NAHMS swine 2012 ELISA test

A total amount of 5730 serum samples from 206 farms were collected from USA National Animal Health Monitoring System's (NAHMS) Swine 2012 study which focus on operations with 100 or more pigs. Serum samples were taken from up to 30 grower/finisher market pigs (20-32 weeks of age) from 13 states. In our study, about 15 samples from each farm were collected, but some farms have more than 15 serum samples. Considering some farms have serum samples less than 15, if the sample number

is 8 or more, all the serums in that farm were collected. If the serum samples were less than 8, those farms were passed. There are totally 2989 serum samples from 202 farms **(Table 1)**.

Sample level prevalence results from ELISA testing of serum samples for PCV2

Since the non-specific background of ELISA result is about 0.1, the cut-off value is determined as 2 or 3 times of the non-specific background. Based on the cut-off values of 0.2, 0.3, sample prevalence can be calculated by the equation: prevalence= number of seropositive samples/ total sample number *100% **(Table 2)**.

Farm level prevalence results from ELISA testing of serum samples for PCV2

Farms with average OD values of serum samples below the cut-off value are considered negative farms; with average OD values above the cut-off value are considered positive farms **(Table 3).**

Statistical analysis of anti-cap ELISA and anti-rep result

The distribution of OD values for anti-cap ELISA is extremely right skewed **(Figure 4)**. 25%, 50%, 75% and 90% percentile are 0.147, 0.216, 0.334 and 0.520, respectively**.** The mean of all samples is 0.278; the standard deviation is 0.199. After taking log 10 of the x-axis, the values are normally distributed **(Figure 5A).**

For anti-rep ELISA, the distribution of OD values is also right skewed **(Figure 4)**. 25%, 50%, 75% and 90% percentile are 0.111, 0.149, 0.2310 and 0.4045, respectively. The mean of all samples is 0.219; the standard deviation is 0.216. After taking log 10 of the x-axis, the values are normally distributed **(Figure 5A).**

Comparison of Cap and Rep in 2012

To demonstrate whether or not there is a significant difference of OD values tested with anti-cap ELISA and anti-rep ELISA, a paired t-test was performed. The null hypothesis is that there is no difference of these two methods. The alternative hypothesis is that there is a difference of these two methods (two tailed). The paired t-test was performed using GraphPad Prism 6. The t value is 17.05, $p<0.0001$, which indicates the null hypothesis that the mean difference is zero should be rejected. The data provide sufficient evidence that there was a significant mean difference of OD values measured by the capsid-specific ELISA and replicase-specific ELISA methods **(Figure 5B)**.

Relationship of PCV2 anti-cap and anti-rep ELISA results

To analyze the relationship of anti-cap and anti-rep OD values, a scatter plot were exhibited based on the both logged anti-cap and anti-rep OD values **(Figure 6)**. According to the cut-off of 0.2 and 0.3, the scatter plot is divided into four areas a, b, c and d. **a** represent serum samples with seronegative anti-cap and seropositive anti-rep ELISA results, which are experimental errors because it's biological impossible to have presence of Rep but absence of Cap; **b** indicates those pigs were infected with PCV2 because anti-Cap and anti-Rep antibodies are both represent at the same time; **c** stands for those pigs were not vaccinated because there is no Cap-specific antibody; **d** is for serum samples with positive anti-cap but negative anti-rep results, implying those pigs were vaccinated but not infected because anti-Rep antibody is not present. For cut-off as 0.2, there are 135, 805, 1212, 837 serum samples in each area, a, b, c, d, respectively, 4.52%, 26.93%, 40.55%, 28.00% in percentage respectively; for cut-off as 0.3, there are 109, 378, 1952, 550 serum samples in each area a, b, c and d, respectively, 3.65%, 12.65%, 65.31%, 18.40% in percentage respectively.

Comparison of PCV2 infection level in 2006 and 2012

Before vaccine has been widely used as a common prevention against PCV2 in 2006, protection of PCV2 was just focused on facility management and prevention of co-infection with other pathogens. In NAHMS 2006, anti-cap indirect ELISA test was used to give information about infection level together with PCV2 viremia level. Since all

commercial vaccines were designed based on Cap protein, the only structure protein of PCV2, or the inactivated virus, anti-Cap EILSA result can't imply the PCV2 infection level anymore. In NAHMS 2012, anti-rep ELISA was first used to provide information about post-vaccination PCV2 infection level based on the fact that Rep protein is produced when PCV2 virus infected and replicated in host cells. In NAHMS 2006, 78.96% (4756/6234*100%) of all serum samples were tested seropositively using anti-cap ELISA; while in NAHMS 2012, 16.29% to 31.05% of samples were tested seropositively using anti-rep ELISA; but if combined with anti-cap result, as we concluded before, the infection level drops down to 12.65% to 26.93%. Comparing to the result of NAHMS2006, we can thus conclude that PCV2 infection level of 2012 in the field is lower than in 2006.

Figure 1: average and standard deviation of 48 test serums with KLH and blank wells.

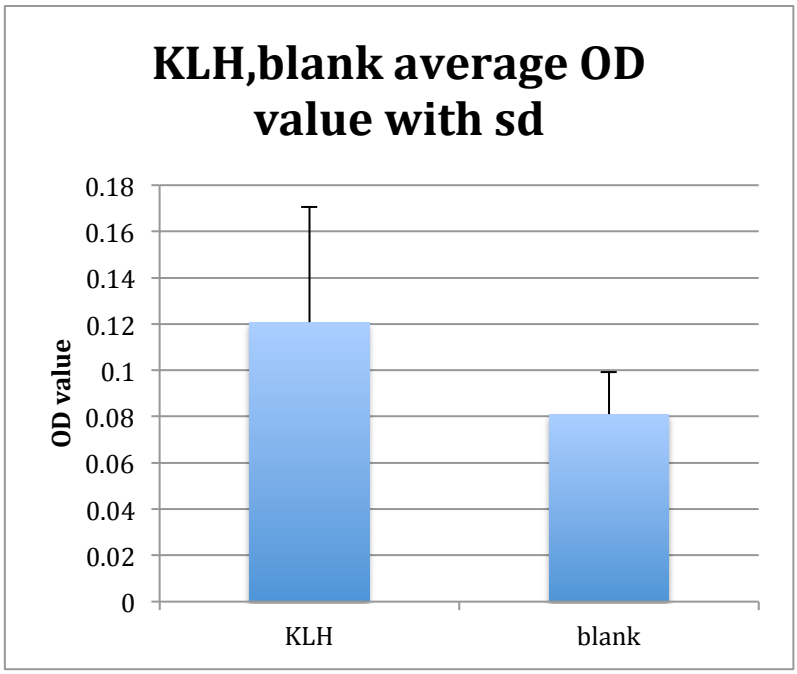
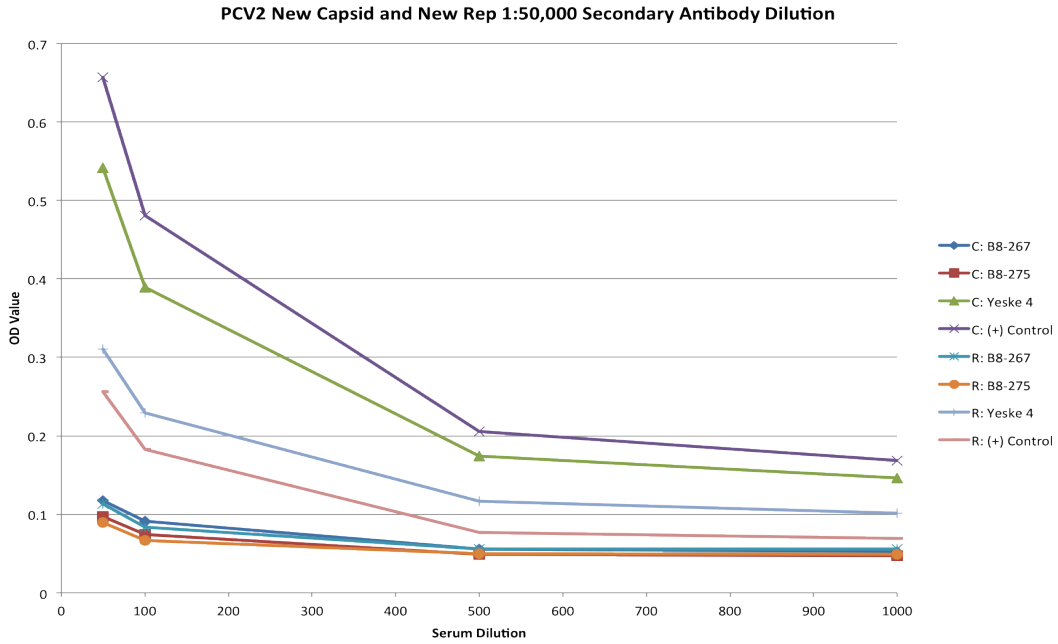

KLH,blank average OD value with sd
OD value
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
KLH
blank

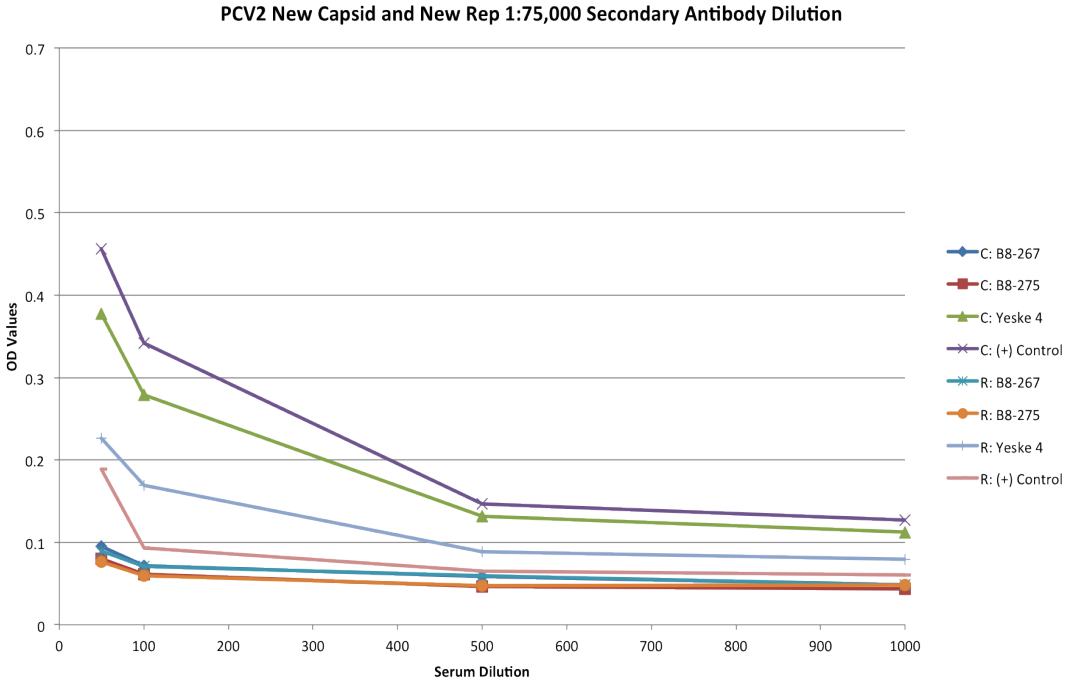
Figure 2: Selection of primary antibodies and secondary antibodies dilutions

Different dilution combinations of primary antibodies and secondary antibodies for both Cap and Rep protein were compared with two seronegative serum samples (B8-267 and B8-275) and two seropositive serum samples (Yeske 4 and positive control). (A) Serum samples were tested in primary antibody dilution of 1:50, 1:100 and 1:1,000 while secondary antibody dilution is 1:50,000. (B) Serum samples were tested in primary antibody dilution of 1:50, 1:100 and 1:1,000 while secondary antibody dilution is 1:75,000. (C) Serum samples were tested in primary antibody dilution of 1:50, 1:100 and 1:1,000 while secondary antibody dilution is 1:100,000.

A


PCV2 New Capsid and New Rep 1:50,000 Secondary Antibody Dilution
OD Value
Serum Dilution
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
200
300
400
500
600
700
800
900
1000
C: B8-267
C: B8-275
C: Yeske 4
C: (+) Control
R: B8-267
R: B8-275
R: Yeske 4
R: (+) Control

B


PCV2 New Capsid and New Rep 1:75,000 Secondary Antibody Dilution
OD Values
Serum Dilution
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
100
200
300
400
500
600
700
800
900
1000
C: B8-267
C: B8-275
C: Yeske 4
C: (+) Control
R: B8-267
R: B8-275
R: Yeske 4
R: (+) Control

C

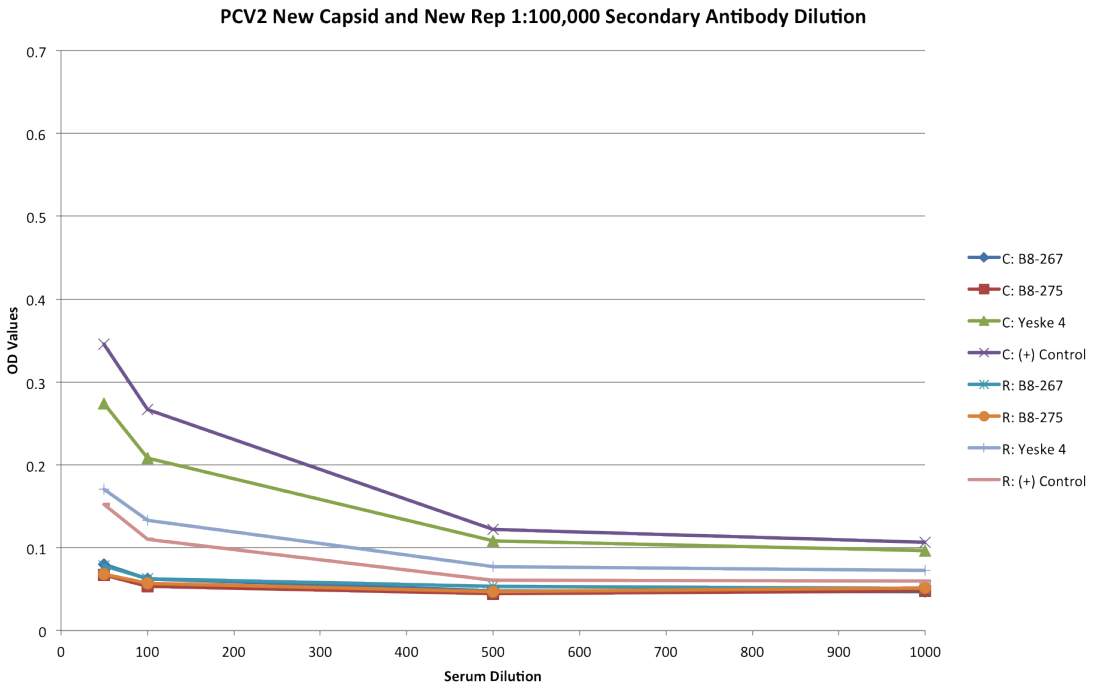

PCV2 New Capsid and New Rep 1:100,000 Secondary Antibody Dilution
OD Values
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
600
700
800
900
1000
Serum Dilution
C: B8-267
C: B8-275
C: Yeske 4
C: (+) Control
R: B8-267
R: B8-275
R: Yeske 4
R: (+) Control

Figure 3: ELISA assay result correlation of serum samples from NAHMS swine 2006 and NAHMS swine 2012.

The OD values of 100 serum samples from NAHMS swine 2006 were tested both by OD values using the new ELISA assay for NAHMS swine 2012 (x-axis) and the previous ELISA assay for NAHMS swine 2006 (y-axis). A linear correlation was measured and a coefficient of determination (R^2) was presented.

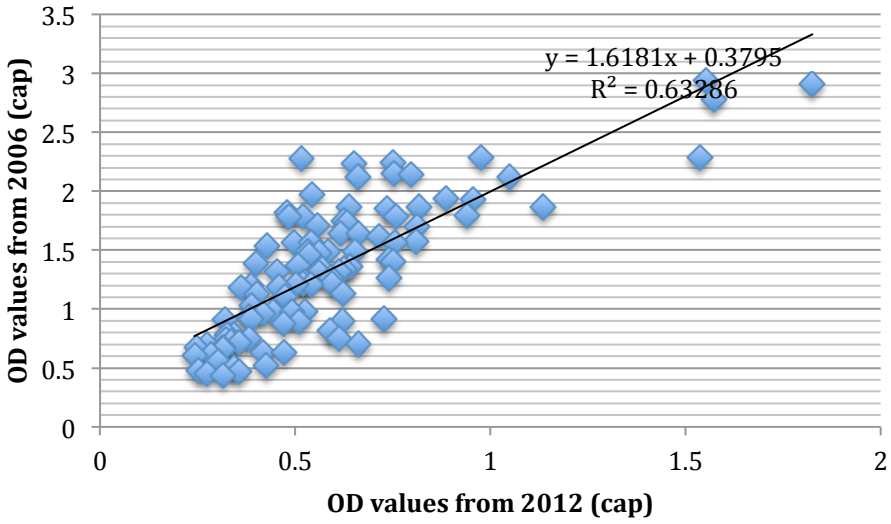

y = 1.6181x + 0.3795
R² = 0.63286
OD values from 2006 (cap)
OD values from 2012 (cap)
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
1
1.5
2

Figure 4: Frequency distribution of PCV2 anti-cap and anti-rep antibody levels.

Distribution of serum samples tested by both cap-specific and anti-rep indirect ELISA was presented by histogram with a unit interval OD value of 0.05.

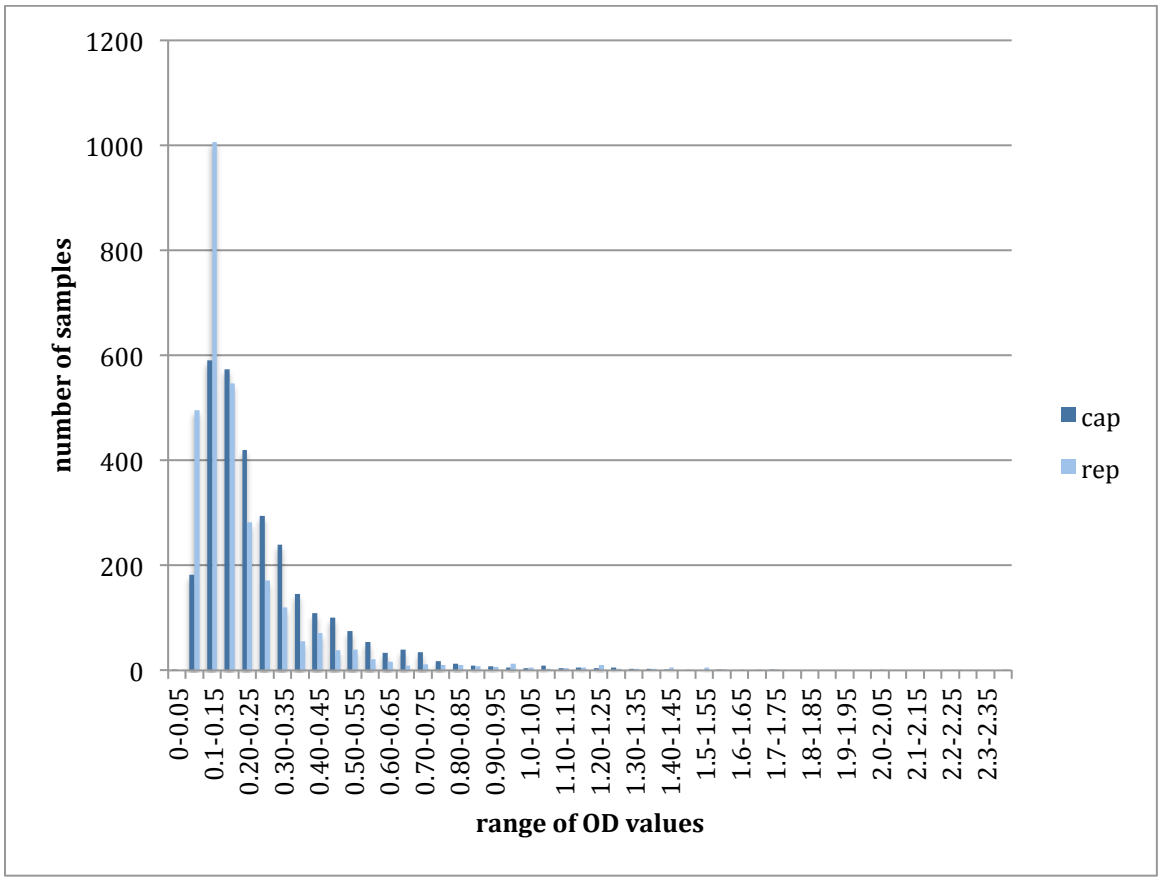
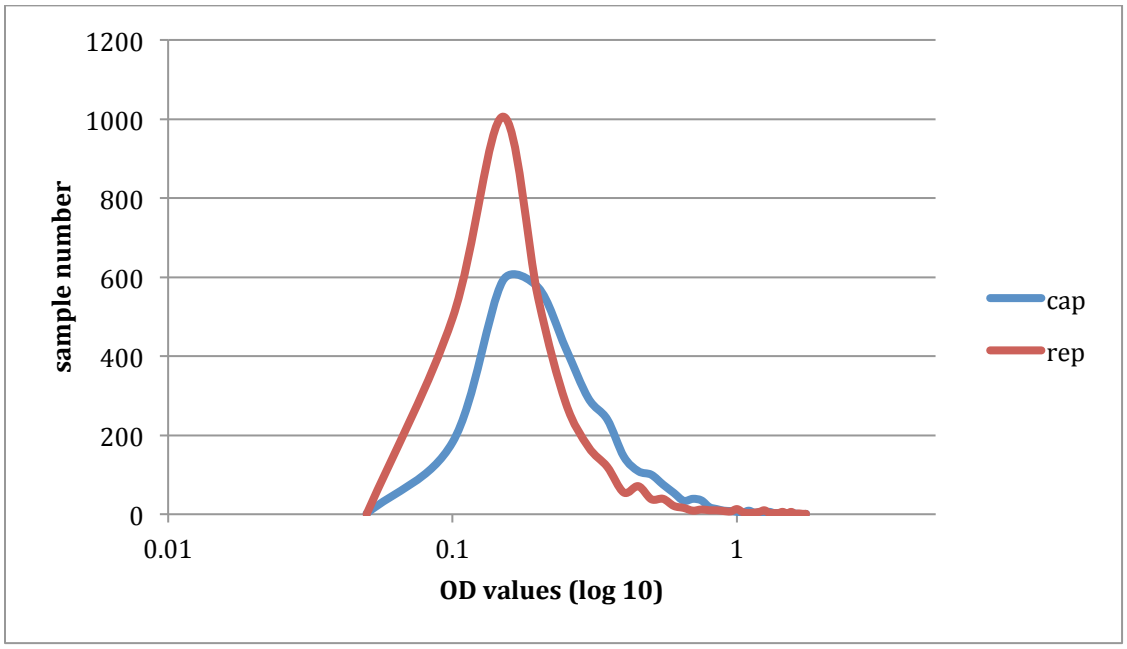

1200
1000
800
600
400
200
0
number of samples
0-0.05
0.1-0.15
0.20-0.25
0.30-0.35
0.40-0.45
0.50-0.55
0.60-0.65
0.70-0.75
0.80-0.85
0.90-0.95
1.0-1.05
1.10-1.15
1.20-1.25
1.30-1.35
1.40-1.45
1.5-1.55
1.6-1.65
1.7-1.75
1.8-1.85
1.9-1.95
2.0-2.05
2.1-2.15
2.2-2.25
2.3-2.35
range of OD values
cap
rep

Figure 5: Comparison of PCV2 anti-cap and anti-rep ELISA results.

(A) Lognormal distributions of anti-cap and anti-rep ELISA result are graphically compared. Blue line represents the distribution of anti-cap ELISA result. Red line represents the distribution of anti-rep ELISA result. (B) Box-plot of anti-cap and anti-rep ELISA OD values (lognormal).

A


1200
1000
800
600
400
200
0
sample number
0.01
0.1
1
OD values (log 10)
cap
rep

B

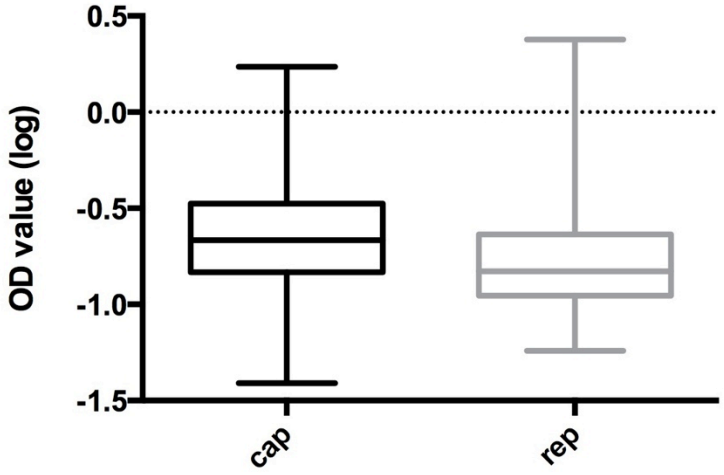
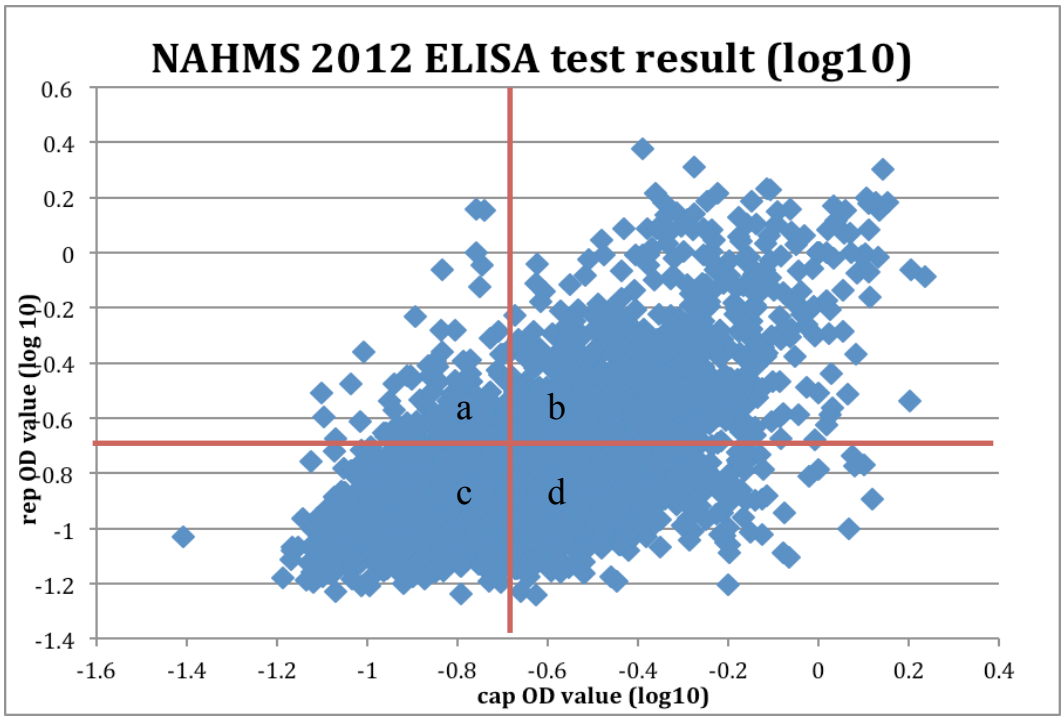

0.5
0.0
-0.5
-1.0
-1.5
OD value (log)
cap
rep

Figure 6: Relationship of PCV2 anti-cap and anti-rep ELISA results.

Relationship of PCV2 anti-cap and anti-rep ELISA results is displayed by two-dimensional scatter plot of logged anti-cap and anti-rep ELISA OD values. Red lines indicate the positive-negative cut-off value for anti-cap and anti-rep ELISA results. Area **a** stands for serum samples with negative anti-cap ELISA results and positive anti-rep ELISA results. Area **b** stands for serum samples with both positive anti-cap and anti-rep ELISA results. Area **c** stands for serum samples with both negative anti-cap and anti-rep ELISA results. Area **d** stands for serum samples with positive anti-cap ELISA results and negative anti-rep ELISA results. All OD values have been taken base-10 log.

A


NAHMS 2012 ELISA test result (log10)
rep OD value (log 10)
cap OD value (log10)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.4
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
a
b
c
d

B

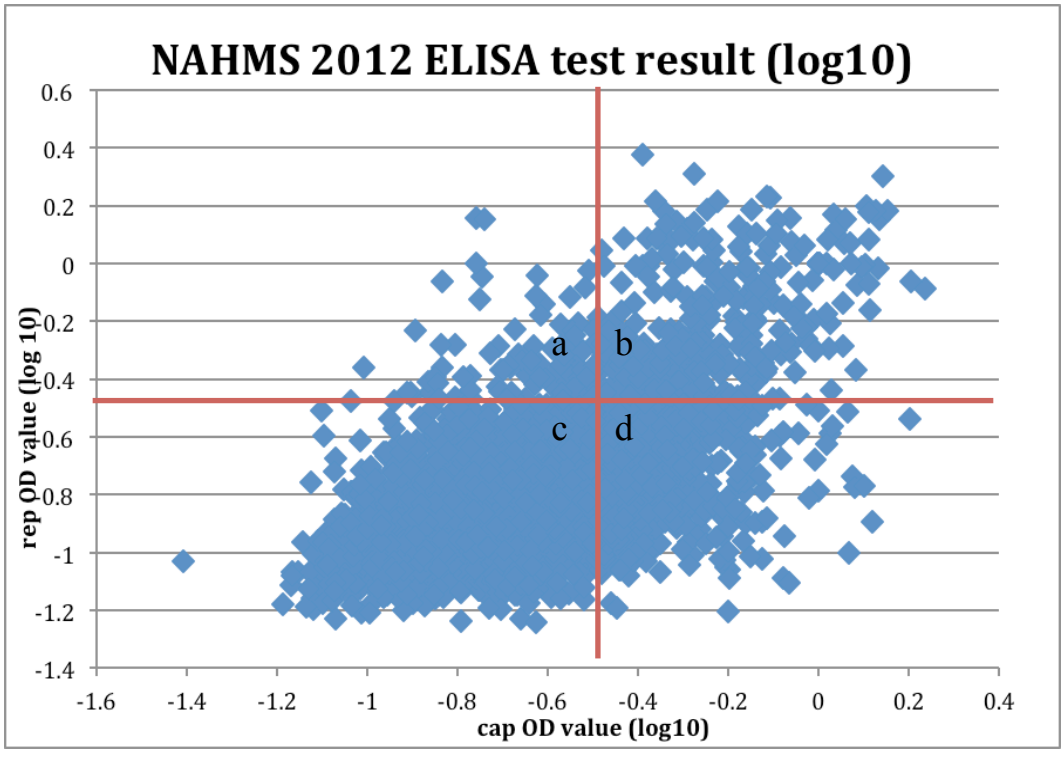

NAHMS 2012 ELISA test result (log10)
rep OD value (log 10)
cap OD value (log10)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
-1.4
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
a
b
c
d

Table 1: Sample and farm level number for PCV2

									Total
Sample number/farm	17	16	15	13	12	10	9	8	
Number of farms	1	5	186	1	2	4	1	2	202
Total number	17	80	2790	13	24	40	9	16	2989

Table 2 Sample prevalence with different cut-off values (%)

Cut	0.2		0.3	
off	-	+	-	+
Cap	45.07	54.93	68.95	31.05
Rep	68.55	31.45	83.71	16.29

Table 3 Farm prevalence with different cut-off values (%)

Cut	0.2		0.3	
off	-	+	-	+
Cap	32.28	67.82	69.31	30.69
Rep	54.46	45.54	84.16	15.84

Chapter 4

Discussion

Since globally emerged as one of the severest swine pathogens in the 1990s, studies of PCV2 have been continually significant in animal health research. Postweaning multisystemic wasting syndrome (PWMS) is the first identified porcine circovirus associated disease (PCVAD) in 1991, but the first commercial vaccine was allowed to use under special license in 2004. PCV2 is considered as the etiological virus of PCVAD, although many other triggers can also have influence on the generation of PCVAD (6). Several vaccine types such as DNA vaccine, recombinant subunit vaccine expressing viral cap protein and inactivated vaccines have been demonstrated to be immunogenic in mice or pigs under experimental condition. PCVAD has also been found effectively prevented and controlled by the widely usage of PCV2 vaccination since 2006 in United States (12), therefore national investigation of the efficacy of PCV2 vaccination is necessary. Yet, to my knowledge, a national-wide investigation of the PCV2 vaccination efficacy has not been studied.

To investigate the prevalence and vaccination status of PCV2 in US swine herd, a total amount of 2989 serum samples from 202 farms of 13 states of United States has been collected and tested serologically using both cap- and rep-specific indirect ELISA. This is

the most up-to-date survey about swine exposure and immune response to PCV2 in the United States.

In present study, in a total of 2989 serum samples, 31.45% - 54.93% were tested positively with anti-cap ELISA, while 16.29% - 31.05% positive with anti-rep ELISA. Since PCV2 was designed based on cap protein or inactivated virus, while rep protein is produced when the virus replicates itself in the cell, different result of anti-cap and anti-rep indirect ELISA can give us both information about vaccination and infection. 12.65% - 26.93% samples with both positive results of anti-cap and anti-rep ELISA indicating that the infection level in those samples is not so high.

In NAHMS 2006 swine study, a total of 6234 serum samples were tested in a capsid-specific indirect ELISA (2). 78.96% of the samples were tested seropositively and 21.04% was seronagetively. Since PCV2 vaccination was not universally applied in field farms before 2006, anti-cap ELISA result can implicate the PCV2 infection level of those farms because it can reflect the antibody level induced by PCV2; while since 2006, PCV2 vaccine was widely used in field condition of United States. Our result suggests there is a reduction in PCV2 infection level comparing to 2006. Our PCR data has also shown only 20% to 25% of PCV2 positive viremia (data not shown), a huge decrease comparing to 82.6% in 2006. It can be suggested that the current PCV2 vaccination has quite good efficacy in preventing PCV2.

In those positive viremia samples, 12% to 15% are PCV2a, 46% to 56% are PCV2b. Rests are unknown strains (data not shown). Interestingly, a similar result in experimental condition suggested a high negative correlation between PCV2 antibody levels and

PCV2b viral load but not for PCV2a, which may reveal a less effective control of PCV2a than PCV2b. Possible explanations may involve the inability of the vaccine to induce effective neutralizing antibodies against PCV2a, even though all commercial vaccines are designed based on PCV2a genotype; or a delayed immune protective response against PCV2a compared to PCV2b.

This investigation doesn't involve PCV1 as NAHMS 2006 did because PCV1 is proved non-pathogenic to swineherds, and recently, PCV2 infection in the pig population has been found to be more common than PCV1 infection (83, 102-104). Result from last national swine study also show a largely absence of PCV1 in finishing swine (100); while PCV2 can cause clinical and subclinical disease contributing the great economic loss in swine industry.

Lack of precise and reproducible animal models for PMWS or PCVAD is one of the main hinders to establishing clear parameters for the experimental evaluation of PCV2 vaccines. Commercial vaccines against PCV2 were initially designed to control PWMS, but they can also be used against other PCVAD. Although prevention of the disease is the main parameter to evaluate the efficacy of vaccination, the prevention of infection, reduce of viremia can also determine the ability of the vaccine to induce immune response to reduce PCV2 transmission.

Efficient PCV2-specific diagnostic tools are necessary to elucidate the infection status. PCV2 antibodies can be accurately detected by IIF and IPMA assays (60, 61, 102, 105, 106), but IIF and IPMA are time and labor consuming for such a large amount of samples. Instead, ELISA is easily to apply and also has a relatively high sensitivity and specificity.

Several modified ELISAs have been developed to specifically detect the PCV2 antibodies. PCV2 viral particles were used in a competitive ELISA (c-ELISA) (62) and a modified PCV2-based ELISA (64), while more recently recombinant capsid protein expressed in baculovirus were used in ORF2 based ELISAs (63, 64), but it is difficult to prepare and purify ELISA antigen from PCV2-infected cells and baculovirus. In NAHMS 2006, it was the first time using a recombinant capsid protein based ELISA to make a national wide serological investigation of PCV2 infection. In our NAHMS 2012, we use both capsid and replicase based ELISA to investigate the infection level and efficacy of vaccination of PCV2.

Since our ELISA assay is different from the one in 2006, maybe reduction in infection level is caused by the different experimental factors comparing to NAHMS 2006. In assay part, we use KLH as our non-specific background instead of haptoglobin that was the one used in NAHMS 2006. But since KLH and haptoglobin are both have no immune response with pigs, they are both good non-background controls. The determination of cut off value in ELISA assay is also a challenge of experimental data analysis. I use the most common method to determine the cut off value, which is two or three times of the non-specific value. The cut-off value may be not so accurate comparing to the real one but the range can give us a quite good trend of the results. Although our indirect ELISA assay has been demonstrated as the most efficient and accurate serological methods for testing antibodies against PCV2 for such a large sample amount, it can just give information in serological fact. Other diagnostic methods such as PCR might provide more information about viremia.

For sample collection part, the number and source of samples are different from NAHMS 2006. Since the samples are all collected voluntarily from farms national wide, the samples in the present study may be from different farms of NAHMS 2006. What's more, the sample size is 6234 in NAHMS 2006 but 2989 in our present study. Those differences may also have influence in our evaluation of the vaccination efficacy. The sample amount of this present study is large enough but since serum samples were collected voluntarily instead of randomly from farms with more than 100 pigs, there may be some restrictions about the national wide conclusions. It will also give us more immunological information if other samples can be provided such as feces or saliva. The information of those farms were confidential, which means no vaccination record or any clinical and subclinical PCVAD was provided to study the relationship between the serological result with the pathological symptoms.

Phylogenetic and evolutionary studies are proved to have increasing importance in molecular epidemiology of infectious pathogens. Although several genotyping studies have been published on PCV2 (8, 27, 107), no evaluation of the utility of the cap or rep genes as molecular markers or assessment of potential PCV2 clonality have been undertaken. This study is the first one use both cap and rep to design an indirect ELISA assay to investigate the infection and vaccination efficacy of PCV2 in US swine herd.

BIBLOGRAPHY

1. Segalés J. The natural history of porcine circovirus type 2: From an inoffensive virus to a devastating swine disease? 2012.
2. Puvanendiran S, Stone S, Yu W, Johnson C, Abrahante J, Jimenez L, et al. Absence of porcine circovirus type 1 (PCV1) and high prevalence of PCV 2 exposure and infection in swine finisher herds. Virus Research. 2011;157(1):92-8.
3. Tischer I, Gelderblom H, Vettermann W, Koch MA. A Very Small Porcine Virus with Circular Single-Stranded-DNA. Nature. 1982;295(5844):64-6.
4. ALLAN G, MCNEILLY F, CASSIDY J, REILLY G, ADAIR B, ELLIS W, et al. PATHOGENESIS OF PORCINE CIRCOVIRUS - EXPERIMENTAL INFECTIONS OF COLOSTRUM DEPRIVED PIGLETS AND EXAMINATION OF PIG FETAL MATERIAL. Veterinary Microbiology. 1995;44(1):49-64.
5. TISCHER I, BODE L, PETERS D, POCIULI S, GERMANN B. DISTRIBUTION OF ANTIBODIES TO PORCINE CIRCOVIRUS IN SWINE POPULATIONS OF DIFFERENT BREEDING FARMS. Archives of Virology. 1995;140(4):737-43.
6. Allan G, McNeilly F, Kennedy S, Daft B, Clarke E, Ellis J, et al. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. Journal of Veterinary Diagnostic Investigation. 1998;10(1):3-10.
7. Meehan BM, McNeilly F, Todd D, Kennedy S, Jewhurst VA, Ellis JA, et al. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol. 1998;79 (Pt 9):2171-9.
8. Hamel A, Lin L, Nayar G. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. Journal of Virology. 1998;72(6):5262-7.
9. Jacobsen B, Krueger L, Seeliger F, Bruegmann M, Segales J, Baumgaertner W. Retrospective study on the occurrence of porcine circovirus 2 infection and associated entities in Northern Germany. Veterinary Microbiology. 2009;138(1-2):27-33.
10. LeCann P, Albina E, Madec F, Cariolet R, Jestin A. Piglet wasting disease. Vet Rec. 1997;141(25):660.
11. Harding JCS, Clark EG. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health and Production. 1997;5(5):201-3.
12. Opriessnig T, Meng X, Halbur P. Porcine circovirus type 2-associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. Journal of Veterinary Diagnostic Investigation. 2007;19(6):591-615.
13. Tischer I, Rasch R, Tochtermann G. Characterization of papovavirus-and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl Bakteriol

Orig A. 1974;226(2):153-67.
14. Meehan BM, Creelan JL, McNulty MS, Todd D. Sequence of porcine circovirus DNA: affinities with plant circoviruses. J Gen Virol. 1997;78 (Pt 1):221-7.
15. Mankertz A, Mankertz J, Wolf K, Buhk HJ. Identification of a protein essential for replication of porcine circovirus. Journal of General Virology. 1998;79:381-4.
16. Mankertz A, Hillenbrand B. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. Virology. 2001;279(2):429-38.
17. Nawagitgul P, Morozov I, Bolin SR, Harms PA, Sorden SD, Paul PS. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol. 2000;81(Pt 9):2281-7.
18. Blanchard P, Mahe D, Cariolet R, Keranflec'h A, Baudouard MA, Cordioli P, et al. Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine. 2003;21(31):4565-75.
19. Liu J, Chen I, Kwang J. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J Virol. 2005;79(13):8262-74.
20. Chaiyakul M, Hsu K, Dardari R, Marshall F, Czub M. Cytotoxicity of ORF3 Proteins from a Nonpathogenic and a Pathogenic Porcine Circovirus. Journal of Virology. 2010;84(21):11440-7.
21. He J, Cao J, Zhou N, Jin Y, Wu J, Zhou J. Identification and Functional Analysis of the Novel ORF4 Protein Encoded by Porcine Circovirus Type 2. Journal of Virology. 2013;87(3):1420-9.
22. Finsterbusch T, Steinfeldt T, Doberstein K, Rodner C, Mankertz A. Interaction of the replication proteins and the capsid protein of porcine circovirus type 1 and 2 with host proteins. Virology. 2009;386(1):122-31.
23. Liu J, Zhu Y, Chen I, Lau J, He F, Lau A, et al. The ORF3 protein of porcine circovirus type 2 interacts with porcine ubiquitin E3 ligase Pirh2 and facilitates p53 expression in viral infection. Journal of Virology. 2007;81(17):9560-7.
24. Timmusk S, Fossum C, Berg M. Porcine circovirus type 2 replicase binds the capsid protein and an intermediate filament-like protein. Journal of General Virology. 2006;87:3215-23.
25. Mankertz A. Molecular interactions of porcine circoviruses type 1 and type 2 with its host. Virus Res. 2012;164(1-2):54-60.
26. Wen L, He K, Yang H, Yu Z, Mao A, Zhong S, et al. Prevalence of porcine circovirus-like agent P1 in Jiangsu, China. Virology Journal. 2011;8.
27. Wen L, He K, Yu Z, Mao A, Ni Y, Zhang X, et al. Complete Genome Sequence of a Novel Porcine Circovirus-Like Agent. Journal of Virology. 2012;86(1):639-.
28. Wen L, He K, Xiao Q, Yu Z, Mao A, Ni Y, et al. A Novel Porcine Circovirus-Like Agent P1 Is Associated with Wasting Syndromes in Pigs. Plos One. 2012;7(8).
29. Segales J, Kekarainen T, Cortey M. The natural history of porcine circovirus type 2: From an inoffensive virus to a devastating swine disease? Veterinary Microbiology. 2013;165(1-2):13-20.
30. Wang F, Guo X, Ge X, Wang Z, Chen Y, Cha Z, et al. Genetic variation analysis of

Chinese strains of porcine circovirus type 2. Virus Research. 2009;145(1):151-6.
31. Cortey M, Olvera A, Grau-Roma L, Segales J. Further comments on porcine circovirus type 2 (PCV2) genotype definition and nomenclature. Veterinary Microbiology. 2011;149(3-4):522-3.
32. Carman S, McEwen B, DeLay J, van Dreumel T, Lusis P, Cai H, et al. Porcine circovirus-2 associated disease in swine in Ontario (2004 to 2005). Can Vet J. 2006;47(8):761-2.
33. Cortey M, Pileri E, Sibila M, Pujols J, Balasch M, Plana J, et al. Genotypic shift of porcine circovirus type 2 from PCV-2a to PCV-2b in Spain from 1985 to 2008. Veterinary Journal. 2011;187(3):363-8.
34. Wiederkehr DD, Sydler T, Buergi E, Haessig M, Zimmermann D, Pospischil A, et al. A new emerging genotype subgroup within PCV-2b dominates the PMWS epizooty in Switzerland. Vet Microbiol. 2009;136(1-2):27-35.
35. Cheung A, Lager K, Kohutyuk O, Vincent A, Henry S, Baker R, et al. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Archives of Virology. 2007;152(5):1035-44.
36. Timmusk S, Wallgren P, Brunborg I, Wikstrom F, Allan G, Meehan B, et al. Phylogenetic analysis of porcine circovirus type 2 (PCV2) pre- and post-epizootic postweaning multisystemic wasting syndrome (PMWS). Virus Genes. 2008;36(3):509-20.
37. Gagnon C, Tremblay D, Tijssen P, Venne M, Houde A, Elahi S. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Canadian Veterinary Journal-Revue Veterinaire Canadienne. 2007;48(8):811-9.
38. Hesse R, Kerrigan M, Rowland RRR. Evidence for recombination between PCV2a and PCV2b in the field. Virus Research. 2008;132(1-2):201-7.
39. Zhai S, Chen S, Wei Z, Zhang J, Huang L, Lin T, et al. Co-existence of multiple strains of porcine circovirus type 2 in the same pig from China. Virology Journal. 2011;8.
40. Harding J, Ellis J, McIntosh K, Krakowka S. Dual heterologous porcine circovirus genogroup 2a/2b infection induces severe disease in germ-free pigs. Veterinary Microbiology. 2010;145(3-4):209-19.
41. Pogranichnyy R, Yoon K, Harms P, Swenson S, Zimmerman J, Sorden S. Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunology. 2000;13(2):143-53.
42. Ouardani M, Wilson L, Jette R, Montpetit C, Dea S. Multiplex PCR for detection and typing of porcine circoviruses. J Clin Microbiol. 1999;37(12):3917-24.
43. Kim J, Chae C. Optimized protocols for the detection of porcine circovirus 2 DNA from formalin-fixed paraffin-embedded tissues using nested polymerase chain reaction and comparison of nested PCR with in situ hybridization. Journal of Virological Methods. 2001;92(2):105-11.
44. Huang C, Hung JJ, Wu CY, Chien MS. Multiplex PCR for rapid detection of pseudorabies virus, porcine parvovirus and porcine circoviruses. Vet Microbiol. 2004;101(3):209-14.
45. Kim J, Han D, Choi C, Chae C. Differentiation of porcine circovirus (PCV)-1 and

PCV-2 in boar semen using a multiplex nested polymerase chain reaction. Journal of Virological Methods. 2001;98(1):25-31.
46. Kim J, Chae C. Multiplex nested PCR compared with in situ hybridization for the differentiation of porcine circoviruses and porcine parvovirus from pigs with postweaning multisystemic wasting syndrome. Can J Vet Res. 2003;67(2):133-7.
47. Brunborg I, Moldal T, Jonassen C. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. Journal of Virological Methods. 2004;122(2):171-8.
48. Olvera A, Sibila M, Calsamiglia M, Segales J, Domingo M. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. Journal of Virological Methods. 2004;117(1):75-80.
49. McNeilly F, Kennedy S, Moffett D, Meehan B, Foster J, Clarke E, et al. A comparison of in situ hybridization and immunohistochemistry for the detection of a new porcine circovirus in formalin-fixed tissues from pigs with post-weaning multisystemic wasting syndrome (PMWS). Journal of Virological Methods. 1999;80(2):123-8.
50. Rosell C, Segales J, Plana-Duran J, Balasch M, Rodriguez-Arrioja G, Kennedy S, et al. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. Journal of Comparative Pathology. 1999;120(1):59-78.
51. Sirinarumitr T, Morozov I, Nawagitgul P, Sorden S, Harms P, Paul P. Utilization of a rate enhancement hybridization buffer system for rapid in situ hybridization for the detection of porcine circovirus in cell culture and in tissues of pigs with postweaning multisystemic wasting syndrome. Journal of Veterinary Diagnostic Investigation. 2000;12(6):562-5.
52. Dvorak CM, Puvanendiran S, Murtaugh MP. Cellular pathogenesis of porcine circovirus type 2 infection. Virus Res. 2013;174(1-2):60-8.
53. Sorden S, Harms P, Nawagitgul P, Cavanaugh D, Paul P. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. Journal of Veterinary Diagnostic Investigation. 1999;11(6):528-30.
54. Kiupel M, Stevenson GW, Mittal SK, Clark EG, Haines DM. Circovirus-like viral associated disease in weaned pigs in Indiana. Vet Pathol. 1998;35(4):303-7.
55. McNeilly F, McNair I, O'Connor M, Brockbank S, Gilpin D, Lasagna C, et al. Evaluation of a porcine circovirus type 2-specific antigen-capture enzyme-linked immunosorbent assay for the diagnosis of postweaning multisystemic wasting syndrome in pigs: comparison with virus isolation, immunohistochemistry, and the polymerase chain reaction. Journal of Veterinary Diagnostic Investigation. 2002;14(2):106-12.
56. Meerts P, Van Gucht S, Cox E, Vandebosch A, Nauwynck H. Correlation between type of adaptive immune response against porcine circovirus type 2 and

level of virus replication. Viral Immunology. 2005;18(2):333-41.
57. TISCHER I, BODE L, APODACA J, TIMM H, PETERS D, RASCH R, et al. PRESENCE OF ANTIBODIES REACTING WITH PORCINE CIRCOVIRUS IN SERA OF HUMANS, MICE, AND CATTLE. Archives of Virology. 1995;140(8):1427-39.
58. Racine S, Kheyar A, Gagnon C, Charbonneau B, Dea S. Eucaryotic expression of the nucleocapsid protein gene of porcine circovirus type 2 and use of the protein in an indirect immunofluorescence assay for serological diagnosis of postweaning multisystemic wasting syndrome in pigs. Clinical and Diagnostic Laboratory Immunology. 2004;11(4):736-41.
59. Kim YH, Kweon CH, Kang SW, Oh YI, Song JY, Lee KK, et al. Vero cells expressing porcine circovirus type 2-capsid protein and their diagnostic application. J Virol Methods. 2013;194(1-2):26-32.
60. Balasch M, Segales J, Rosell C, Domingo M, Mankertz A, Urniza A, et al. Experimental inoculation of conventional pigs with tissue homogenates from pigs with post-weaning multisystemic wasting syndrome. Journal of Comparative Pathology. 1999;121(2):139-48.
61. Ellis J, Krakowka S, Lairmore M, Haines D, Bratanich A, Clark E, et al. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J Vet Diagn Invest. 1999;11(1):3-14.
62. Walker I, Konoby C, Jewhurst V, McNair I, McNeilly F, Meehan B, et al. Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2. Journal of Veterinary Diagnostic Investigation. 2000;12(5):400-5.
63. Blanchard P, Mahe D, Cariolet R, Truong C, Le Dimna M, Arnauld C, et al. An ORF2 protein-based ELISA for porcine circovirus type 2 antibodies in post-weaning multisystemic wasting syndrome. Veterinary Microbiology. 2003;94(3):183-94.
64. Nawagitgul P, Harms P, Morozov I, Thacker B, Sorden S, Lekcharoensuk C, et al. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked Immunosorbent assays for detection of antibodies to PCV. Clinical and Diagnostic Laboratory Immunology. 2002;9(1):33-40.
65. Liu C, Ihara T, Nunoya T, Ueda S. Development of an ELISA based on the baculovirus-expressed capsid protein of porcine circovirus type 2 as antigen. Journal of Veterinary Medical Science. 2004;66(3):237-42.
66. Shang S, Li Y, Guo J, Wang Z, Chen Q, Shen H, et al. Development and validation of a recombinant capsid protein-based ELISA for detection of antibody to porcine circovirus type 2. Research in Veterinary Science. 2008;84(1):150-7.
67. Perez-Martin E, Grau-Roma L, Argilaguet J, Nofrarias M, Escribano J, Gomez-Sebastian S, et al. Development of two Trichoplusia ni larvae-derived ELISAs for the detection of antibodies against replicase and capsid proteins of porcine circovirus type 2 in domestic pigs. Journal of Virological Methods. 2008;154(1-2):167-74.
68. Chae C. Commercial porcine circovirus type 2 vaccines: Efficacy and clinical application. Veterinary Journal. 2012;194(2):151-7.
69. Blanchard P, Mahe D, Cariolet R, Keranflec'h A, Baudouard M, Cordioli P, et al. Protection of swine against post-weaning multisystemic wasting syndrome (PMWS)

by porcine circovirus type 2 (PCV2) proteins. Vaccine. 2003;21(31):4565-75.
70. Gerber P, Johnson J, Shen H, Striegel D, Xiao C, Halbur P, et al. Association of concurrent porcine circovirus (PCV) 2a and 2b infection with PCV associated disease in vaccinated pigs. Research in Veterinary Science. 2013;95(2):775-81.
71. Fenaux M, Opriessnig T, Halbur P, Elvinger F, Meng X. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. Journal of Virology. 2004;78(12):6297-303.
72. Fenaux M, Opriessnig T, Halbur P, Meng X. Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. Journal of Virology. 2003;77(20):11232-43.
73. Fort M, Sibila M, Allepuz A, Mateu E, Roerink F, Segales J. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine. 2008;26(8):1063-71.
74. Opriessnig T, O'Neill K, Gerber PF, de Castro AMMG, Gimenez-Lirola LG, Beach NM, et al. A PCV2 vaccine based on genotype 2b is more effective than a 2a-based vaccine to protect against PCV2b or combined PCV2a/2b viremia in pigs with concurrent PCV2, PRRSV and PPV infection. Vaccine. 2013;31(3):487-94.
75. Kixmoller M, Ritzmann M, Eddicks M, Saalmuller A, Elbers K, Fachinger V. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine. 2008;26(27-28):3443-51.
76. Fachinger V, Bischoff R, Ben Jedidia S, Saalmuller A, Elbers K. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine. 2008;26(11):1488-99.
77. Segales J, Urniza A, Alegre A, Bru T, Crisci E, Nofrarias M, et al. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) improves clinical, pathological and virological outcomes in postweaning multisystemic wasting syndrome affected farms. Vaccine. 2009;27(52):7313-21.
78. Pejsak Z, Podgorska K, Truszczynski M, Karbowiak P, Stadejek T. Efficacy of different protocols of vaccination against porcine circovirus type 2 (PCV2) in a farm affected by postweaning multisystemic wasting syndrome (PMWS). Comp Immunol Microbiol Infect Dis. 2010;33(6):e1-5.
79. Horlen K, Dritz S, Nietfeld J, Henry S, Hesse R, Oberst R, et al. A field evaluation of mortality rate and growth performance in pigs vaccinated against porcine circovirus type 2. Javma-Journal of the American Veterinary Medical Association. 2008;232(6):906-12.
80. Fraile L, Grau-Roma L, Sarasola P, Sinovas N, Nofrarias M, Lopez-Jimenez R, et al. Inactivated PCV2 one shot vaccine applied in 3-week-old piglets: Improvement of production parameters and interaction with maternally derived immunity. Vaccine. 2012;30(11):1986-92.
81. Shen H, Beach N, Huang Y, Halbur P, Meng X, Opriessnig T. Comparison of commercial and experimental porcine circovirus type 2 (PCV2) vaccines using a

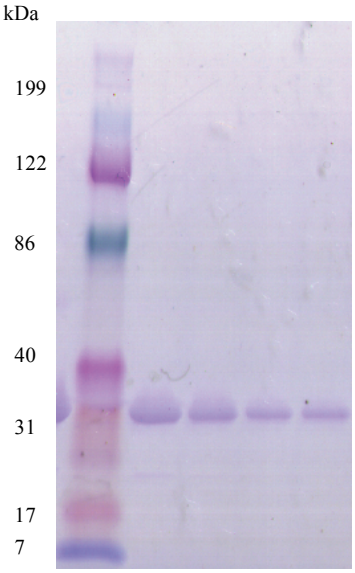
triple challenge with PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV). Vaccine. 2010;28(37):5960-6.
82. Opriessnig T, Patterson AR, Madson DM, Pal N, Halbur PG. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine. 2009;27(7):1002-7.
83. Liu Q, Wang L, Willson P, Babiuk L. Quantitative, competitive PCR analysis of porcine circovirus DNA in serum from pigs with postweaning multisystemic wasting syndrome. Journal of Clinical Microbiology. 2000;38(9):3474-7.
84. Opriessnig T, Madson D, Prickett J, Kuhar D, Lunney J, Elsener J, et al. Effect of porcine circovirus type 2 (PCV2) vaccination on porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 coinfection. Veterinary Microbiology. 2008;131(1-2):103-14.
85. Opriessnig T, Patterson A, Elsener J, Meng X, Halbur P. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clinical and Vaccine Immunology. 2008;15(3):397-401.
86. Madson D, Patterson A, Ramamoorthy S, Pal N, Meng X, Opriessnig T. Effect of Porcine Circovirus Type 2 (PCV2) Vaccination of the Dam on PCV2 Replication In Utero. Clinical and Vaccine Immunology. 2009;16(6):830-4.
87. Magar R, Larochelle R, Thibault S, Lamontagne L. Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. Journal of Comparative Pathology. 2000;123(4):258-69.
88. Bolin S, Stoffregen W, Nayar G, Hamel A. Postweaning multisystemic wasting syndrome induced after experimental inoculation of cesarean-derived, colostrum-deprived piglets with type 2 porcine circovirus. Journal of Veterinary Diagnostic Investigation. 2001;13(3):185-94.
89. McIntosh K, Harding J, Parker S, Ellis J, Appleyard G. Nested polymerase chain reaction detection and duration of porcine circovirus type 2 in semen with sperm morphological analysis from naturally infected boars. Journal of Veterinary Diagnostic Investigation. 2006;18(4):380-4.
90. Segalés J. Porcine circovirus diseases. 2005.
91. Harding JCS, Clark EG, Strokappe JH, Willson PI, Ellis JA. Postweaning multisystemic wasting syndrome: Epidemiology and clinical presentation. Swine Health and Production. 1998;6(6):249-54.
92. Segales J, Sitjar M, Domingo M, Dee S, Del Pozo M, Noval R, et al. First report of post-weaning multisystemic wasting syndrome in pigs in Spain. Veterinary Record. 1997;141(23):600-1.
93. Chae C. Porcine circovirus type 2 and its associated diseases in Korea. Virus Res. 2012;164(1-2):107-13.
94. Segales J. Porcine circovirus type 2 (PCV2) infections: Clinical signs, pathology and laboratory diagnosis. Virus Research. 2012;164(1-2):10-9.
95. Grau-Roma L, Fraile L, Segales J. Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. Veterinary

Journal. 2011;187(1):23-32.
96. Rose N, Opriessnig T, Grasland B, Jestin A. Epidemiology and transmission of porcine circovirus type 2 (PCV2). Virus Research. 2012;164(1-2):78-89.
97. Vidigal PM, Mafra CL, Silva FM, Fietto JL, Silva Junior A, Almeida MR. Tripping over emerging pathogens around the world: a phylogeographical approach for determining the epidemiology of Porcine circovirus-2 (PCV-2), considering global trading. Virus Res. 2012;163(1):320-7.
98. Ramirez-Mendoza H, Martinez C, Mercado C, Castillo-Juarez H, Hernandez J, Segales J. Porcine circovirus type 2 antibody detection in backyard pigs from Mexico City. Research in Veterinary Science. 2007;83(1):130-2.
99. Ge XW, F. Guo, X. Yang, H. Porcine circovirus type 2 and its associated diseases in China. Virus Res. 2012;164(1-2):100-6.
100. Puvanendiran S, Stone S, Yu W, Johnson CR, Abrahante J, Jimenez LG, et al. Absence of porcine circovirus type 1 (PCV1) and high prevalence of PCV 2 exposure and infection in swine finisher herds. Virus Res. 2011;157(1):92-8.
101. Gnanandarajah JS, Dvorak CMT, Johnson CR, Murtaugh MP. Presence of free haptoglobin alpha 1S-subunit in acute porcine reproductive and respiratory syndrome virus infection. Journal of General Virology. 2008;89:2746-53.
102. Magar R, Muller P, Larochelle R. Retrospective serological survey of antibodies to porcine circovirus type 1 and type 2. Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire. 2000;64(3):184-6.
103. Ellis JA, Bratanich A, Clark EG, Allan G, Meehan B, Haines DM, et al. **Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired postweaning multisystemic wasting syndrome**. J Vet Diagn Invest. 2000;12(1):21-7.
104. Larochelle R, Antaya M, Morin M, Magar R. Typing of porcine circovirus in clinical specimens by multiplex PCR. Journal of Virological Methods. 1999;80(1):69-75.
105. Raye W, Muhling J, Warfe L, Buddle J, Palmer C, Wilcox G. The detection of porcine circovirus in the Australian pig herd. Australian Veterinary Journal. 2005;83(5):300-4.
106. Vicente J, Segales J, Hofle U, Balasch M, Plana-Duran J, Domingo M, et al. Epidemiological study on porcine circovirus type 2 (PCV2) infection in the European wild boar (Sus scrofa). Veterinary Research. 2004;35(2):243-53.
107. Kim HK, Luo Y, Moon HJ, Park SJ, Keum HO, Rho S, et al. Phylogenetic and recombination analysis of genomic sequences of PCV2 isolated in Korea. Virus Genes. 2009;39(3):352-8.
108. Wei C, Zhang M, Chen Y, Xie J, Huang Z, Zhu W, et al. Genetic evolution and phylogenetic analysis of porcine circovirus type 2 infections in southern China from 2011 to 2012. Infect Genet Evol 2013 Jul;17:87-92.

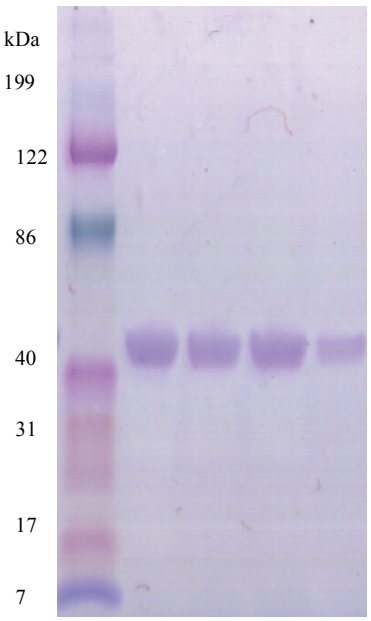
APPENDIX 1

Figure: Gel pictures of cap (A) and rep (B) protein

A.



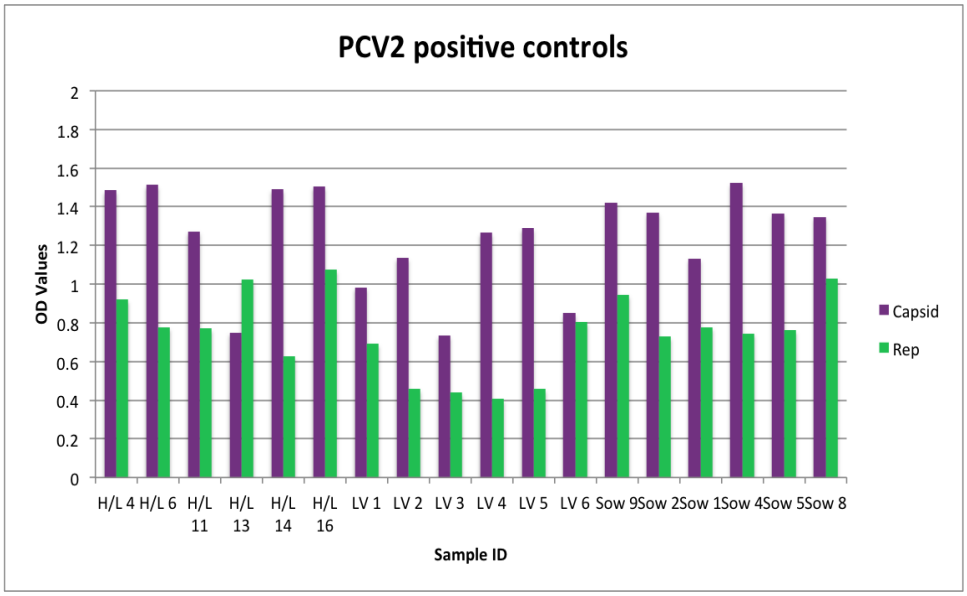
B.



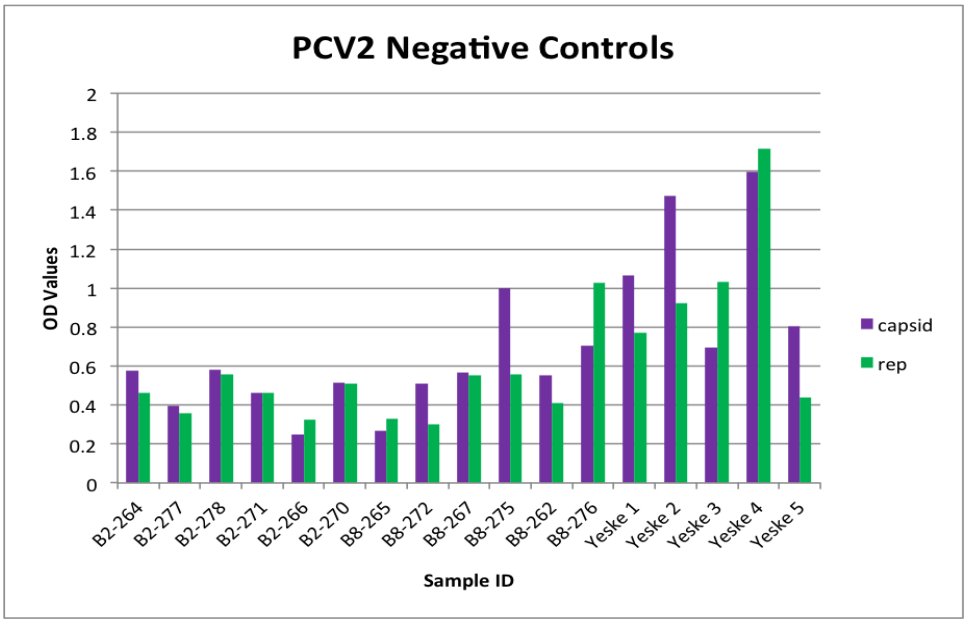
APPENDIX 2

Figure: Selection positive control from previous PCV2 study with cut-off of 0.4. H/L 16, Sow8, Sow 9 strains were chosen and pooled together as the positive control (A). B8-265 was chosen as the negative control (B).

A.


PCV2 positive controls
OD Values
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H/L 4
H/L 6
H/L 11
H/L 13
H/L 14
H/L 16
LV 1
LV 2
LV 3
LV 4
LV 5
LV 6
Sow 9
Sow 2
Sow 1
Sow 4
Sow 5
Sow 8
Sample ID
Capsid
Rep

B


PCV2 Negative Controls
OD Values
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B2-264
B2-277
B2-278
B2-271
B2-266
B2-270
B8-265
B8-272
B8-267
B8-275
B8-262
B8-276
Yeske 1
Yeske 2
Yeske 3
Yeske 4
Yeske 5
Sample ID
capsid
rep

APPENDIX3

Table: Sample and farm level prevalence from both anti-cap and anti-rep ELISA testing for PCV2 of different cut off values

a. Sample and farm level prevalence with cut-off of 0.1

	Sample level prevalence				Farm level prevalence			
	Cap		Rep		Cap		Rep	
	N	Percent	N	Percent	N	Percent	N	Percent
Negative	183	6.12	496	16.59	1	0.5	11	5.4
Positive	2806	93.88	2493	83.41	201	99.5	190	94.6
Total	2989	100	2989	100	202	100	202	100

b. Sample and farm level prevalence with cut off of 0.2

	Sample level prevalence				Farm level prevalence			
	Cap		Rep		Cap		Rep	
	N	Percent	N	Percent	N	Percent	N	Percent
Negative	1347	45.07	2049	68.55	65	32.18	110	54.5
Positive	1642	54.93	940	31.45	137	67.82	92	45.5
Total	2989	100	2989	100	202	100	202	100

c. Sample and farm level prevalence with cut off of 0.25

	Sample level prevalence				Farm level prevalence			
	Cap		Rep		Cap		Rep	
	N	Percent	N	Percent	N	Percent	N	Percent
Negative	1767	59.12	2331	77.99	101	50	147	72.77
Positive	1222	40.88	658	22.01	101	50	55	27.23
Total	2989	100	2989	100	202	100	202	100

d. Sample and farm level prevalence with cut off of 0.3

	Sample level prevalence				Farm level prevalence			
	Cap		Rep		Cap		Rep	
	N	Percent	N	Percent	N	Percent	N	Percent
Negative	2061	68. 95	2502	83. 71	140	69.31	170	84.16
Positive	928	31. 05	487	16. 29	62	30.69	32	15.84
Total	2989	100	2989	100	202	100	202	100

f. Sample and farm level prevalence with cut off of 0.35

	Sample level prevalence				Farm level prevalence			
	Cap		Rep		Cap		Rep	
	N	Percent	N	Percent	N	Percent	N	Percent
Negative	2301	76. 98	2622	16.59	156	77.23	179	88.62
Positive	688	23. 02	367	83.41	46	22.78	23	11.39
Total	2989	100	2989	100	202	100	202	100