RESEARCH REPORT
SWINE HEALTH

Title: Dissemination of PCV2 viral particles from sow to piglets - **NPB # 09-184**

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II. Industry Summary.

PCVAD is a disease of finishing pigs that is controlled by vaccination. It is effective in prevention of PCVAD and reducing the level of PCV2 in serum, but does not eliminate infection. Since nearly all of the finishing herds in the United States are vaccinated, we are inadvertently providing a large-scale selective pressure on PCV2 for new strains that grow better in the presence of an anti-PCV2 vaccine response. At the same time, we are maintaining a continuous source of infection from shedding pigs, and raising the fixed cost of pork production. Our goal is to understand mechanisms of immunity that will help make vaccines that completely eliminate PCV2 infection. To achieve this goal we need a challenge model that mimics infection in the field. Contrary to the widespread belief that infection occurs at 10-15 weeks of age, we thought that infection might occur early in life since nearly all market-age pigs appeared to be infected. Here, we examined PCV2 and anti-PCV2 immune status in sows, piglets, and the farrowing environment from 6 sow farms in Minnesota, Iowa, and Indiana. The vast majority of sows are viremic for PCV2 even though they have high levels of PCV2-specific antibodies. The virus is everywhere; colostrum, oral fluids, feces, skin, crate bars, and floor. Cleaning with disinfectants reduces viral load, but does not eliminate it. Seventy-eight percent (78%) of piglets were born viremic, and the rest are infected soon after. Our findings, that sows of all parities are persistently infected in the presence of an anti-PCV2 immune response and that piglets are infected in utero or during nursing, show that PCV2 infection occurs near the beginning of life and is lifelong. Thus, a relevant challenge model for vaccine development should use infected pigs.

III. Keywords.

Porcine Circovirus 2, PCV2, Farrowing, Sow, Piglet, PCVAD

IV. Scientific Abstract.

Porcine circovirus 2 (PCV2) infection, which is necessary for PCV-associated disease (PCVAD), is widespread in swine farms throughout the United States. Vaccination of pigs, frequently around the time of weaning, has been effective in preventing PCVAD and reducing the level of PCV2 in serum, but it does not eliminate infection. Since nearly all of the finishing herds in the United States are vaccinated, we are inadvertently providing a large-scale selective pressure on PCV2 for new strains that grow better in the presence of an anti-PCV2 vaccine response. Improving vaccine efficacy against infection requires a better understanding of when and how pigs are exposed to and become productively infected with PCV2. Infection is thought to occur in finishers at 10-15 weeks of age, when they become viremic. However, infection of sows may be prevalent, and PCV2 is known to be shed in colostrum, milk, and feces, and the virus is stable in the environment. Therefore, we hypothesized that piglets are exposed to PCV2 at or before birth and throughout the suckling period, and that anti-PCV2 antibodies in colostrum might suppress viremia. We examined sows, pre-suckling piglets, and the farrowing environment of farrowing farms for PCV2 virus and PCV2-specific antibodies. PCV2 DNA was observed in serum, oral fluids, and colostrum of sows, even though antibody levels were high in serum and colostrum. PCV2 DNA was detected in tissues from stillborn pigs and mummified fetuses, and in serum of pre-suckling liveborn piglets, indicating that piglets are readily infected with PCV2 in utero. PCV2 also was detected on the axillary skin of presucking pigs and sows and on farrowing crate surfaces. Farrowing crate disinfection procedures reduced, but did not eliminate detection of viral DNA. Overall, PCV2 is widely distributed in sow farms and is transmitted to piglets in utero and after birth. The presence of high levels of antibodies does not resolve infection in sows, but may suppress infection in piglets since absence of viremia is commonly observed in nursery-age pigs.

V. Introduction.

Porcine circovirus 2 (PCV2) infection is widespread in swine farms throughout the United States. PCV2 is the causative agent for PCVAD (PCV-associated disease). Vaccination of pigs, frequently around the time of weaning, has been effective in preventing PCVAD and reducing the level of PCV2 in serum, but it does not eliminate infection. Since nearly all of the finishing herds in the United States are vaccinated, we are inadvertently providing a large-scale selective pressure on PCV2 for new strains that grow better in the presence of an anti-PCV2 vaccine response. Our long-term goal is to achieve completely effective immune elimination and prevention of PCV2 infection. To achieve this goal, we would like to understand the mechanisms of immunity against PCV2 and to use this information to improve vaccine design and administration.

Development of a research model for elucidation of the mechanisms of anti-PCV2 immunity requires the knowledge of when and how pigs are exposed to and become productively infected with PCV2. Infection in finishers appears to occur at 10-15 weeks of age, when they become viremic. But nearly all finishing age pigs are infected with PCV2, suggesting that gilts and sows also are infected. Since PCV2 is known to be shed in colostrum, milk, and feces, and the virus is stable in the environment, piglets must be exposed to PCV2 throughout the suckling period and perhaps during gestation. Immune sows also deliver anti-PCV2 antibodies via milk, which may be able to prevent or control PCV2 infection in newborn piglets.

In this study we have examined sows, pre-suckling piglets, and the crate environment of farrowing farms for PCV2 virus and PCV2-specific antibodies. We have observed PCV2 DNA in all sample types, however the number of animals that are positive per farm and the viral levels in those animals varies between animals and between farms. PCV2-specific antibodies have been detected at high levels in both sow serum and colostrum. PCV2 is shown to be widely distributed in sow farms and is present in sows even in the presence of high levels of antibodies.

VI. Objectives.

The project objectives stated below take the first step in addressing the hypothesis that *piglets are infected with PCV2 from sows during the nursing period*. We will examine farrowing sows and their environment under field conditions to:

1. **Determine the PCV2 status of sows at farrowing** by examining serum, oral fluids and fecal samples for levels of PCV2-specific antibodies and PCV2 DNA. *Working hypothesis*: Sows are infected with PCV2, irrespective of viremia status. The research questions here are (1) what is the level of PCV2 viremia in commercial sow herds, (2) does PCV2 replicate in and shed from sites not monitored in serum, (3) what is the effect of parity on PCV2 status, (4) what is the genetic diversity of PCV2 in sow herds, and (5) what are the levels of anti-PCV2 antibodies in serum, feces, oral fluids, colostrum, and milk?

2. **Examine possible routes of dissemination of PCV2 from farrowing sows** through the detection of PCV2 DNA from fluids, secretions, and the environment. *Working hypothesis:* Sows secrete PCV2 virus into the environment through feces and milk. The research questions here are (1) what are the sources of PCV2 exposure to newborn piglets and what is their relative importance, (2) are piglets infected in utero, and (3) does maternal immunity suppress or prevent infection in piglets?

The intended outcomes of the project are the following:
– Quantitative PCV2 infection and shedding status of farrowing gilts and sows.
– Effect of parity on PCV2 infection and shedding status.
– Routes of dissemination of PCV2 from farrowing and nursing sows.
– Genetic identity and relationships of PCV2 isolates from sows.
– Levels of anti-PCV2 antibodies in sows and secreted into colostrum and milk.
– Level of PCV2 in the piglet environment.
– Genetic identities of environmental PCV2.
– Quantitative PCV2 infection status of piglets presuckling.
– Quantitative PCV2 infection status of piglets at weaning. (future study)
– Association of virus and antibody levels in sows with infection and antibody status in piglets at weaning. (future study)
– Genetic identities of weaned pig PCV2 isolates. (future study)
– Intended follow-up of weaned pigs through finishing to assess viremic status and viral genetic identity (future study).

V. Materials and Methods.

On farm sampling

A total of 6-14 sows, which farrowed on the same day, were sampled at each farm. A total of 6 farms were sampled (Table 1). On the day of farrowing, up to 5 newborn piglets from each sow were bled pre-suckling in clot tubes. Serum was isolated, aliquoted, and refrigerated (4°C) for short-term storage or frozen (-20°C) for long-term storage. Colostrum was sampled from sows within 12h after farrowing. The aqueous fraction was stored frozen. Sow feces were collected in a whirlpac bag and stored frozen. A small sample of placenta was excised and frozen. Environmental samples (sow saliva, axillary skin swabs from sows and piglets, floor, crate, and bar samples) and a uterine sample were collected using a dry swab, which was then placed in 500ul PBS and refrigerated for 12-24h. The swab was spun to remove all liquid, which was then frozen at -20°C. Uterine swabs were enclosed in a plastic sleeve during sampling to ensure that only the uterine environment was swabbed. An unused farrowing room was sampled after washing and after disinfection. A swiffer-type pad was used to wipe along the concrete aisle, floor aisle, crate floor, crate bar, or crate walls. Each pad was mixed with 30ml PBS in a plastic zipper bag, the liquid was squeezed out of the pad and the liquid was stored at -20°C.

Approximately 3 days after farrowing, blood was harvested from sows in clot tubes and serum was isolated and frozen at -20°C. Sow feces and environmental samples were obtained as on the day of farrowing.

Stillborn or mummified fetuses and piglets that died within the first 3 days after farrowing (up to 2 per sow) were necropsied and tissue samples were harvested and frozen at -20°C. A second sample of tissue, approximately $1cm^3$, was placed into 500ul RNAlater, stored at 4°C overnight and then frozen at -20°C. The samples acquired included axillary skin swabs, thoracic fluid, ILN, MLN, liver, lung, spleen, kidney, jejunum, ileum, colon, tonsil, and heart. A sample from the abdominal area of the mummified fetuses also was obtained and frozen.

DNA isolation and determination of viral copy number

Total DNA (including PCV2 DNA) from serum, colostrum, thoracic fluid, and swabs was isolated using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA). For each liquid sample, 200ul of starting material was used and DNA isolated following the manufacturer's protocol with a final elution in 50ul of sterile water. From feces, total DNA was isolated using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. A total of 200 mg of feces was used as the starting material and the final elution was in 200ul of sterile water. The Qiagen DNeasy blood and tissue mini kit (Qiagen, Valencia, CA) was used for DNA isolation from frozen tissues. DNA was isolated from approximately 20mg of tissue (10mg for spleen) following the manufacturer's protocol and eluted twice with 200ul of sterile water for a total elution volume of 400ul. All isolated DNA samples were stored at -20°C.

Viral copy number was determined by a PCV2ab real-time PCR assay using the Quanta Fast SYBR master mix (Quanta BioSciences, Inc., Gaithersburg, MD), 375nM primers (forward 5'GCCAGAATTCAACCTTMACYTTYC3' and reverse 5'GCGGTGGAATGMTGAGATT3') and 5ul of isolated DNA in a 20ul reaction. DNA isolated from feces, tissues, and uterine fluid was diluted 1:10 before use. Reactions were run in a Stratagene MX3000 machine with the following cycling conditions; 95°C activation for 1min followed by 40 cycles of 95°C for 3s and 59°C for 25s, followed by a dissociation step to determine the melting temperature of the product. A standard curve and positive and negative controls for both PCV2a and PCV2b were run on each plate to determine the number of DNA copies.

ELISA analysis of PCV2-specific antibodies

Serum, colostrum, saliva, and fecal samples were analyzed for PCV2 capsid-specific antibodies using a standard ELISA protocol. Antibodies from fecal samples were extracted by incubating feces with extraction buffer (5% NFDM plus protease inhibitors) and spinning down the particulates. The collected fluid was stored in aliquots at -20°C until use. ELISA wells were coated overnight with 200ng PCV2 capsid protein in carbonate buffer at pH 9.6, washed with PBST at pH 7.4, and blocked for 2h with 5% non-fat dry milk in PBST at pH 9.6. Plates were washed and then incubated for 1h at room temperature with primary antibody using the following dilutions; serum 1:50, colostrum IgG 1:5000, colostrum IgM and IgA 1:50, oral fluids 1:3, and feces were serially diluted from 1:16.7 to 1:984,000. Plates were washed and secondary antibody, swine IgG-, IgM-, or IgA-HRP (Bethyl Laboratories, Inc, Montgomery, TX), was added and incubated for 1h at room temperature at the following dilutions; serum and feces 1:100,000, colostrum and oral fluids 1:25,000. Plates were then washed and TMB peroxidase substrate solutions (KPL, Inc., Gaithersburg, MD) were added to each well for 15 minutes to develop color. Development was quenched by adding 1M phosphoric acid and plates were read at 450nm using a Thermo Max microplate reader (Molecular Devices, Sunnyvale, CA).

VIII. Results.

Farms examined

Six sow farms in Minnesota, Iowa, and Indiana were studied. Specific information for each farm and the number of pigs examined from each farm is shown in Table 1. A total of 59 sows and 281 piglets were examined. Three of these farms, 1, 3, and 5, were of high health and were PRRSV-free. Two of the farms, 3 and 5, vaccinated farrowing sows 3-4 weeks before farrowing with PCV2 vaccine.

Table 1. Farm information.

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6
Size	3500 Sow	2400 Sow	1300 Sow	1700 Sow	2000 Sow	3200 Sow
Farm type	Farrow	Farrow to finish	Farrow to finish	Farrow	Farrow	Farrow
Parities	0-2	mixed	mixed	mixed	mixed	mixed
Air handling	Filtered	Not filtered	Not filtered	Not filtered	Not filtered	Filtered
Health status	PRRS negative	PRRS +	PRRS negative	PRRS stable	PRRS negative	PRRS stable
PCV2 vaccination			Vaccinate Sows		Vaccinate Sows	
# sows sampled	14	10	9	11	9	6
# piglets sampled	67	50	45	46	43	30

Virus and antibody levels in sows

In sows, serum was examined for both virus and for PCV2-specific antibodies. The percentage of animals that were viremic varied by farm (Figure 1), but every farm had viremic sows. Parity was not a factor affecting viremia levels. The presence of PCV2 in sows suggests that virus could be transmitted to piglets in utero or at birth.

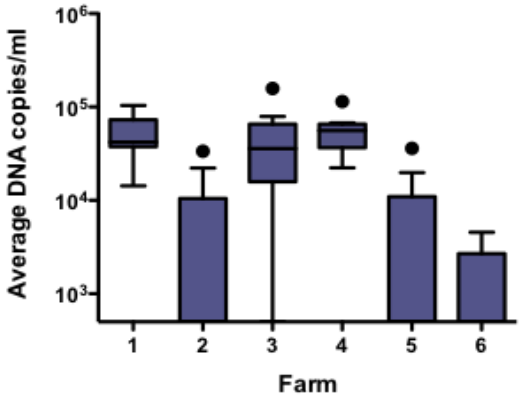

Figure 1 – PCV2 viremia in sows. Serum was harvested from sows 2-3 days post-farrowing. DNA was isolated and quantitative real-time PCR was used to examine serum for PCV2–specific DNA.

Sow serum was also examined for PCV2-specific antibodies. All of the sows contained high levels of PCV2-specific antibodies as determined by IgG ELISA (Figure 2A) as shown on a per farm basis. The two vaccinated farms, 3 and 5, had universally high levels of antibodies in all animals. Even in the presence of high levels of antibodies, virus was still present in serum. PCV2-specific antibody levels were also examined in colostrum, oral fluids, and feces from sows. Colostral antibodies were present at extremely high levels between farms (Figure 2B). Serum and colostral IgG levels correlated very nicely with each other. IgG and IgA antibodies were observed at low levels in the oral fluids of sows (data not shown). IgM antibodies were not present in detectible amounts in either oral fluids or colostrum. No significant differences were observed

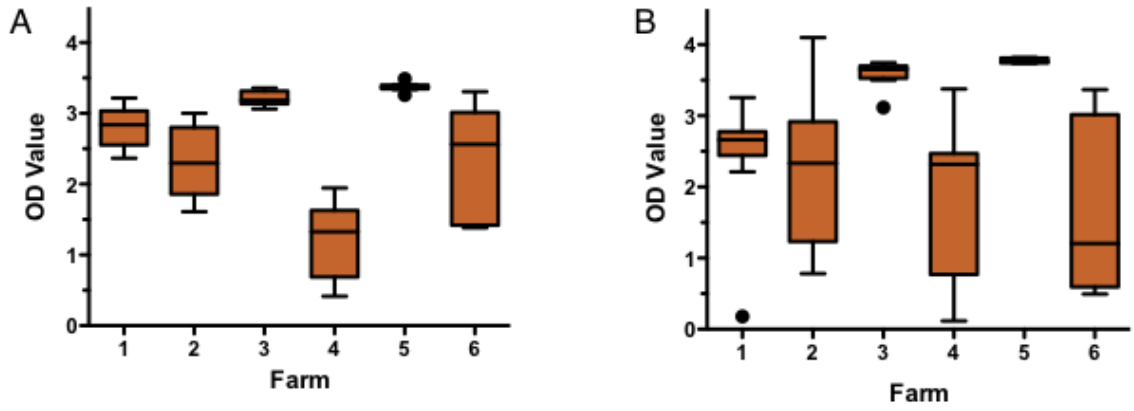

Figure 2. PCV2-specific antibody levels in sows. Sow serum (A) and colostrum (B) were examined for the presence of anti-PCV2 capsid antibodies using ELISA.

between day 1 and day 3 oral fluid IgG or IgA antibodies within farms. However, antibody levels were significantly different between farms, with Farms 3 and 5 (vaccinated sows) having the highest levels of antibodies. PCV2 specific antibodies were not observed in fecal samples, however total antibodies were observed (data not shown).

Viral shedding into the environment

Oral fluids, colostrum, and feces from sows were examined to determine if virus was present in secretions, which could then be shed into the farrowing environment. Oral fluids and feces were sampled on the day of farrowing and 3 days post-farrowing. The majority of animals were positive for both colostrum and oral fluids with viral levels varying between animals and between farms (Figure 3A and B). In feces, 42% of samples were PCV2 positive.

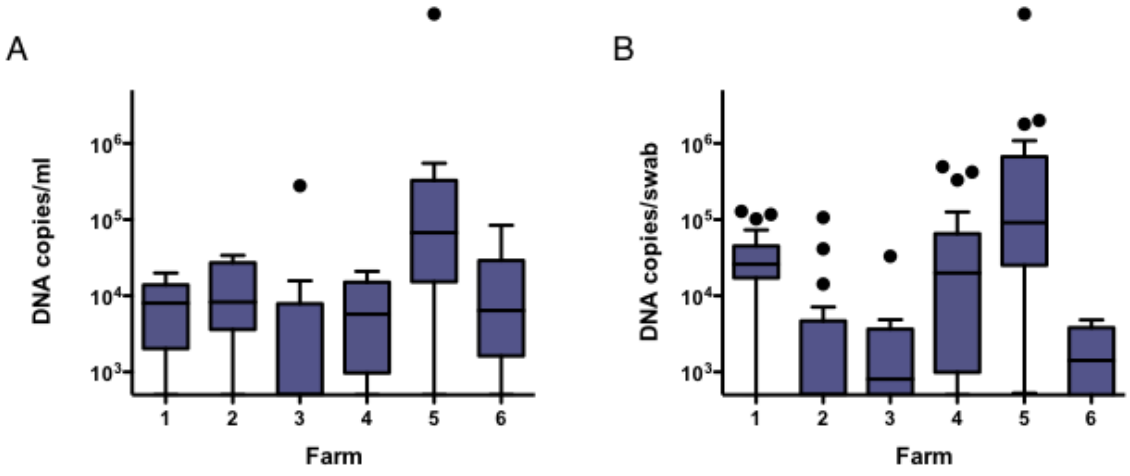

Figure 3. PCV2 viral levels in sow secretions. Sow colostrum (A) and oral fluids (B) were examined for PCV2 DNA using quantitative real-time PCR.

Environmental contamination by PCV2

The farrowing environment was examined for PCV2 DNA in order to determine if piglets could be infected with PCV2 through environmental exposure. The farrowing crate floor, bars, and the skin of the sows were examined for PCV2 DNA on they day of farrowing and post-farrowing for all farms (Figure 4A). Variation in viral levels may be due to sampling procedures or the cleanliness of the farrowing crate when the animals were first moved in.

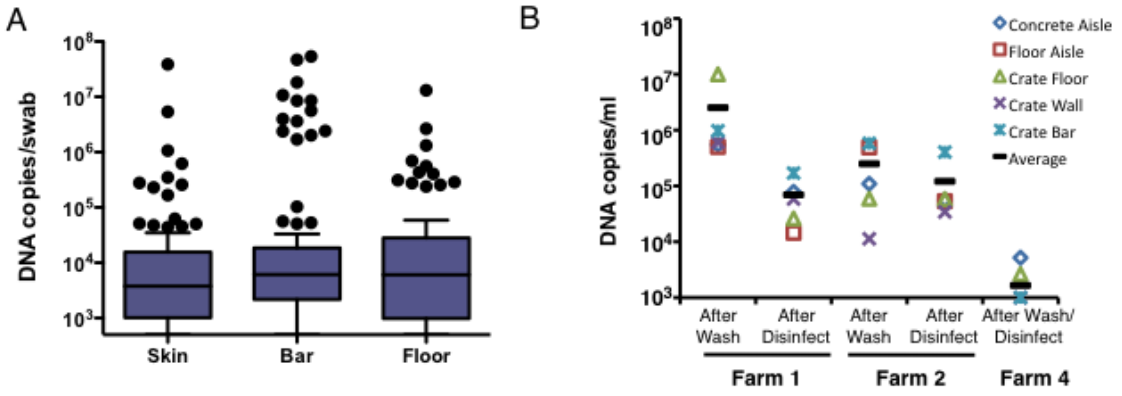

Figure 4. PCV2 viral levels in the farrowing environment. (A) Swabs from the skin of farrowing sows, the crate bar, and the crate floor were examined on the day of farrowing and 2-3 days post-farrowing for PCV2 DNA using quantitative real-time PCR. (B) Empty farrowing rooms from three separate farms were sampled after power-washing and decontamination procedures. PCV2 DNA levels were quantitated using real-time PCR.

At three of the farms an empty farrowing room was sampled after power washing and then again after decontamination. Farm 4 was power washed and decontaminated in the same step and sampled only after decontamination. Farms 1 and 4 used the decontaminant Synergize and Farm 2 used Virkon-6. PCV2 DNA was present at high levels after power washing and was decreased, but still present, after decontamination (Figure 4B).

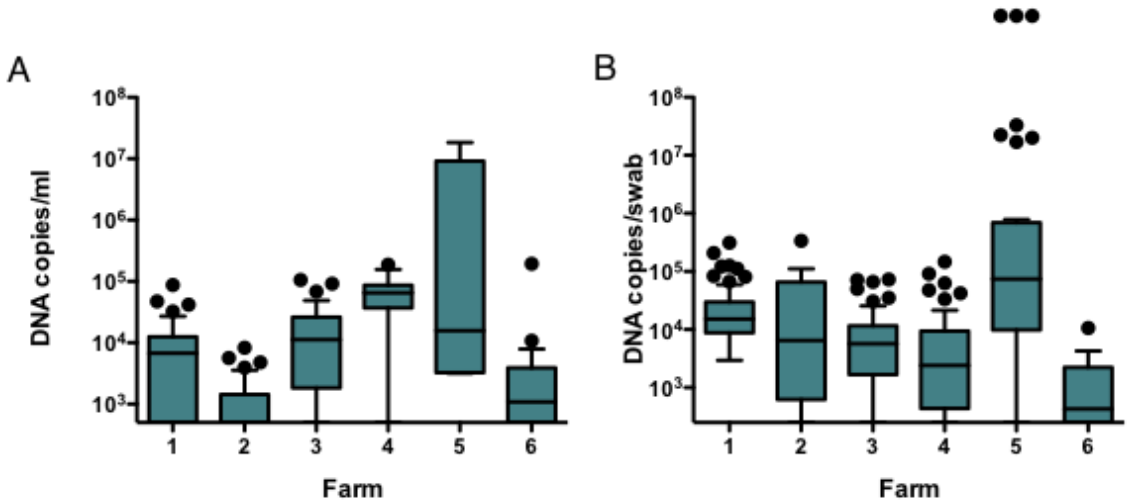

Figure 5. PCV2 presence in pre-suckling piglets. Serum (A) and skin swabs (B) from piglets were obtained pre-suckling and levels of PCV2 DNA were examined using quantitative real-time PCR.

Virus levels in piglets

Pre-suckling piglet serum was obtained from newborn piglets and examined for PCV2 DNA using real-time PCR. A total of 78% of piglets were born viremic (Figure 5A). There was no correlation to parity of the sow or to the levels of viremia observed in the sow. Non-viremic sows were present in farms 2, 3, 5, and 6, however all of these sows, except 1 from farm 2, gave birth to at least one viremic piglet. This suggests that although these animals may not be viremic, they must have virus replicating elsewhere in their bodies. In fact, even in the non-viremic sows, PCV2 was sometimes observed in saliva, colostrum, or feces. PCV2-specific antibody levels for piglets, pre-suckling, were examined and the majority of piglets were negative (data not shown). This confirms that the serum samples were obtained pre-suckling. However, in a few piglets (<5%), PCV2-specific antibodies were detected. This observation suggests that in utero infection of piglets may have stimulated an active immune response during fetal development.

A swab of the skin of the axillary space of the piglets foreleg was examined for PCV2 DNA. This is expected to be the cleanest part of skin on the newborn piglets and least likely to come into contact with the farrowing crate environment in the first moments after birth. Thus, samples here would help determine if piglets were exposed to PCV2 in utero or during the birthing process as opposed to after birth. The majority of piglets (93%) were positive for PCV2 DNA from skin swabs (Figure 5B), suggesting they came into contact with PCV2 before or during birth. Viral levels from piglet skin swabs were higher than that from the sow skin swabs suggesting that virus on piglet skin was not from the same source as the sows, the farrowing environment. There was no correlation between virus levels on the skin of the piglets and the parity of the sow, the skin swabs from the sow, or viral levels in sow serum.

Piglets that were mummified, stillborn, or died within 3 days after birth were examined for the presence of PCV2 DNA in various tissues. The majority of the mummified fetuses showed high levels of virus in the tissue tested. The highest viral levels from stillborn piglets were observed in lung, liver, and tonsil samples. The highest percent of PCV2 positive samples was observed in lung tissues from stillborn piglets (Figure 6). The viral levels in different tissues varied between farms and no single tissue stood out as much higher than any other which would have suggested a major site of viral replication. Liveborn piglets that died within 3 days after birth had low levels of virus, if any, present in the tissues, however virus was detectible on the skin and in thoracic fluid of most liveborn piglets.

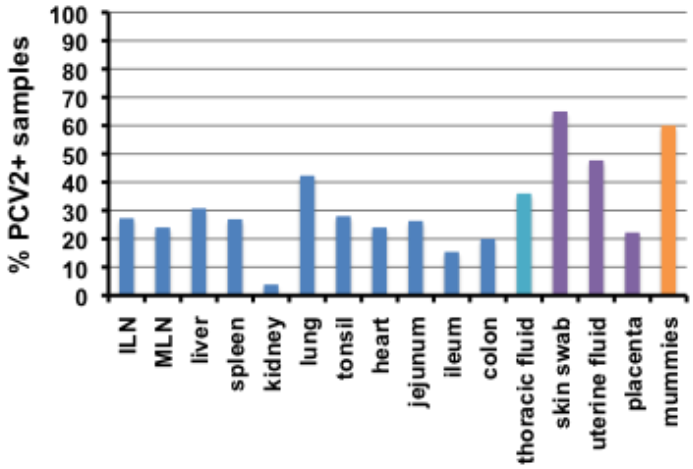

Figure 6. PCV2 presence in reproductive tissues and stillborn and mummified fetuses. Tissue samples and swabs from placenta, stillborn and mummified fetuses, uterine swabs, and stillborn skin swabs and thoracic fluid were examined for PCV2 DNA levels using quantitative real-time PCR.

IX. Discussion.

A survey of the PCV2 status of farrowing sows, their environment, and their piglets has revealed that PCV2 is widely distributed on sow farms. Thus, it appears that sows are constantly exposed to PCV2 and piglets are exposed to PCV2 in utero, at the time of birth, and constantly thereafter from contact with the sow and the environment. Sows are viremic for PCV2 at the same time that they have high levels of anti-PCV2 antibodies. Since infection and antibody levels were independent of parity, we conclude that the immune

response does not eliminate infection in sows. Thus, PCV2 infection appears to be truly persistent and lifelong. In this study, different farm types had varying levels of prevalence and viral loads.

Sows have been shown to be constantly shedding virus into the environment (Segalés et al, 2005, Shibata et al. 2006), which has been confirmed by our study. PCV2 DNA is ubiquitous throughout the farrowing environment, even after disinfection. Thus, even a low level of infectious virus would allow for the infection and constant challenge of newborn piglets. In our study we found that 78% of piglets (pre-suckling) are born viremic, suggesting they were infected in utero. High levels of anti-PCV2 antibodies were present in colostrum, which may protect piglets against disease, or infection, or both. It is not known if colostral antibodies can prevent PCV2 infection, especially during the constant viral exposure present in the farrowing room, and perhaps in nurseries after weaning. Interestingly, PCVAD is often observed around 10-15 weeks of age, shortly after the decline of maternal antibodies that usually occurs at 8-10 weeks. It appears that maternal antibodies are able to control viremic infection until they decrease, at which time viral infection of lymphoid tissues becomes apparent through the release of virus into the bloodstream.

In utero infection also was demonstrated in stillbirths and mummies, in which PCV2 infection may have contributed to the failure of developing fetuses to survive. Levels of PCV2 infection were greatly decreased by 2-3 days of age, indicating that the PCV2 infection was controlled or eliminated by maternal antibodies. It remains to be determined if exposure and infection early in life is completely controlled by passive maternal immunity, or only masked and viremia becomes evident when maternal immunity wanes after weaning.

All farms surveyed were PCV2 positive in sows, piglets, and the environment. Our observation that a high health herd in an air-filtered environment has high levels of virus, low levels of antibodies, and no PCVAD, confirms that PCV2 is not the only factor necessary for disease causation. This setting may be a good model to examine additional factors necessary for expression of PCVAD.

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