

**IS PORCINE PERIWEANING FAILURE-TO-THRIVE SYNDROME AN INFECTIOUS
DISEASE?**

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Abstract

Porcine Periweaning Failure-to-Thrive syndrome (PFTS) is a clinical syndrome of newly weaned pigs with unknown etiology and characterized by anorexia, lethargy and progressive debilitation. The hypothesis of this thesis is that PFTS is an infectious disease. Investigation in an index farm affected by PFTS from Saskatchewan Canada ruled out most common swine pathogens as the etiology and identified several lesions that were consistent across many cases. A larger study including multiple farms in North America was then undertaken. A total of 8 farms were investigated, within which 5 met the clinical definition of PFTS. Gross and histological examinations were performed on 8 case and 4 control pigs on each farm. Detection of relevant porcine pathogens, complete blood count, serum chemistry, and serum cytokine analysis were performed on each pig. Thymic atrophy, superficial gastritis and small intestinal villous atrophy were significantly more prevalent in case pigs compared to control pigs. All case pigs had at least two of these three lesions. All case and control pigs were negative for porcine reproductive and respiratory syndrome virus, swine influenza virus and were free of porcine circovirus associated diseases. Although several pathogens, such as porcine cytomegalovirus, haemagglutinating encephalomyelitis virus, porcine enteric calicivirus, group A rotavirus, enteroviruses and *Cystoisospora suis* were detected in some of the case and control pigs, none were associated with clinical status. Clinical pathology findings of case pigs was consistent with anorexia and dehydration, such as increases in haematocrit, blood urea, serum bilirubin, albumin, beta-hydroxybutyrate and decreases in blood glucose, calcium and phosphorous. Case pigs had similar levels to IL1- β than control pigs, which suggested that PFTS was not a result of excessive cytokines. In subsequent experiments, a snatched-farrowed porcine-colostrum-deprived (SF-pCD)

pig model was developed and tissue homogenates were used to inoculate SF-pCD pigs in an attempt to reproduce the clinical signs of PFTS. The SF-pCD pigs were immunologically characterized and shown to be suitable for inoculation studies. However, inoculation of tissue homogenate from PFTS pigs failed to reproduce the clinical signs of PFTS in SF-pCD pigs. All together, PFTS is a clinical syndrome with consistent pathological and serum analytical changes among affected pigs. Despite the efforts of this research to establish an infectious etiology, there is a lack of evidence that PFTS is an infectious disease.

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To my mother, who is in progressive sickness at the time of writing this thesis;

To my love, Vivi Hui Pan, who continues to love and support me all through the years of our
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To the God revealed in Jesus Christ, who already loved me when I was still an enemy of Him.

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List of abbreviations

ACoV-1	Alphacoronavirus 1
AST	Aspartate transaminase
BCoV-1	Betacoronavirus 1
BHB	Beta-hydroxybutyrate
CBC	Complete blood cell count
CDCD	Caesarean-derived colostrum-deprived
CPE	Cytopathic effect
CTRL	Control
CV	Coefficient of variation
DON	Deoxynivalenol
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunoassay
FLUAV	Influenza A virus
FMIA	Fluorescent microsphere immunoassay
GEE	Generalized Estimating Equations
GLDH	Glutamate dehydrogenase
Hct	Haematocrit
HEV	Haemagglutinating encephalomyelitis virus
Hgb	Haemoglobin
Hh	<i>Helicobacter-heilmannii</i> -like organism
Hp	<i>Helicobacter-pylori</i> -like organism

IM	Intramuscularly
INOC	Inoculation
IP	Intraperitoneally
Mhyo	<i>Mycoplasma hyopneumoniae</i>
OTA	Ochratoxin
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PCV	Pack cell volume
PCMV	Porcine cytomegalovirus
PCV2	Type 2 porcine circovirus
PCVAD	Porcine circovirus-associated diseases
PDS	Prairie Diagnostic Services
PECV	Porcine enteric calicivirus
PEV	Porcine enterovirus
PFTS	Periweaning failure-to-thrive syndrome
PMWS	Porcine post-weaning multisystemic wasting syndrome
PRRS	Porcine reproductive and respiratory syndrome
RV-A	Group A rotavirus
SF-pCD	Snatched-farrowed porcine-colostrum-deprived
SIV	Swine influenza virus
SPF	Specific pathogen free
SuHV-2	Suid herpesvirus 2
TDS	Total dissolved solids

TGEV	Transmissible gastroenteritis virus
TTV	Torque Teno virus

1. Literature review

1.1. Introduction of PFTS

1.1.1. Brief history: from “starve-outs” to PFTS

After weaning, pigs enter to a new phase of life. Their diet changes from sow’s milk to solid feed. The abrupt transition from mainly liquid diet to solid feed poses a challenge in that they must learn to adapt this change. As a result, postweaning anorexia and a postweaning growth check commonly occur in weaned pigs, but most pigs begin to eat within 24 hours postweaning.¹⁷ However, this may or may not happen successfully. It is well recognized that some pigs seem to transition poorly to solid feed and subsequently die from starvation. A weaned pig with this condition is called a postweaning “starve-out” and it has been known to occur in the swine industry for a long time. The exact prevalence of postweaning starve-outs in a batch of nursery pigs is not known, but understandably it must be lower than typical nursery mortality. Nursery mortality rates vary among farms, but taking a recent study as an example, nursery mortality with standard farm management was reported as 3.6% (172440 pigs from 32 sites).¹⁴⁷ Thus it can be estimated that the batch prevalence of starve-outs is lower than 3.6%. It may be that early weaning (i.e. weaned at 2 weeks of age) is a risk factor for pigs not adapting to solid feed, however, weaning older pigs does not eliminate starve-outs.⁵⁹ Starve-outs are visually thin and hairy with depressed mental status and a gaunt abdomen, demonstrate no activity around a feeder and are dehydrated.⁵⁹ Antibiotic treatment is not effective and it has been suggested that the first 30 hours postweaning is critical in identifying starve-outs because most of the pigs should have consumed solid feed by that time.⁵⁹ Potential underlying factors that may cause or predispose pigs to overt postweaning starvation has not been investigated, possibly due to the low

prevalence of the condition and that it has been accepted as a “normal” phenomenon in commercial pork production. This however has changed.

In 2008, two groups independently reported clinical syndromes in pigs which appeared to be “outbreaks” of postweaning starve-outs affecting up to 15% of nursery pigs.^{42,57} As a result, the nursery mortality rate in affected farms was elevated above historical averages. The clinical syndrome waxed and waned and was not predictable in affected farms. In an affected farm in western Canada, efforts to resolve the problem including adjustments to ventilation, feed and water sources, antibiotic medication, adjustment of water nipples and use of electrolytes were ineffective. Desiccation of the farrowing and nursery rooms with aerosolized hydrated lime appeared to be the only effective method of reducing mortality, but the evidence was equivocal. The partial success of aerosolized lime did, however, provide some evidence that an infectious organism might play a role in these outbreaks.

Since 2008, this syndrome was increasingly recognized and discussed. Different names were used to describe the syndrome, including “postweaning catabolic syndrome”,⁴² “postweaning wasting-catabolic syndrome”,^{51,57} “failure to thrive syndrome”²⁰⁰ and “postweaning fading pig-anorexia syndrome”.¹⁶⁹ To facilitate investigation and communication, a standardized name was proposed at the 2010 International Pig Veterinary Society Congress (Vancouver, BC, Canada). A number of researchers, diagnosticians and swine practitioners from North America met, discussed and reached the consensus that “Porcine Periweaning Failure-to-Thrive Syndrome (PFTS)” would be a suitable name for this syndrome, because it reflects the age of onset and clinical presentation.⁷⁹ The word “periweaning” was chosen because: 1) the age of onset seemed to be right at weaning; 2) it should not be confused with Porcine Postweaning Multisystemic Wasting Syndrome (PMWS) caused by porcine circovirus type 2 (PCV2); and 3) the origin or

etiology may begin during the suckling phase.

1.1.2. Clinical presentation

Clinical signs originally reported were mostly non-specific (i.e. anorexia and lethargy) and consistent with starve-outs at the individual pig level.^{42,57} Diarrhea was inconsistently present.⁵⁷ Dehydration, depression, sneezing and standing but not willing to move were later also listed as most frequent clinical signs. Additionally, Dr. Steven Henry, Abilene, Kansas, who was the veterinarian for several farms affected by PFTS, made an interesting and important observation that some affected pigs in all affected farms demonstrated repetitive oral behaviour such as licking, chewing or chomping, which was subsequently thought to be characteristic of PFTS.⁷⁹

1.1.3. Possible causes of PFTS

The etiology of PFTS is not presently known but theories have been suggested. PCV2, Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and *Mycoplasma hyopneumoniae* were thought to be unlikely causative agents of PFTS.⁵⁷ Calicivirus was observed in feces of some affected pigs by electron microscopy in one of the original reports, which might be responsible for small intestinal villous atrophy and diarrhea.⁵⁷ Haemagglutinating Encephalomyelitis Virus (HEV), a group 2 coronavirus that causes Vomiting and Wasting Disease was detected in some PFTS pigs,¹⁶⁹ but data showing association between HEV and PFTS was not presented.¹⁶⁹ Dr. Steven Henry, who contributed substantially to the early description of PFTS, suggested that Porcine Cytomegalovirus (PCMV) might be responsible for the sneezing and suppurative rhinitis frequently encountered in PFTS pigs (2010, personal communication). He later proposed that vitamin D deficiency may be the cause of PFTS, but subsequently discovered it to be widespread in USA swine nurseries (personal communication, 2011). Use of a different breed of boar was

associated with the “disappearance” of PFTS in one farm (Pittman, personal communication 2011). When the author presented descriptions of PFTS to different audiences, frequently encountered suggestions of etiologies also included mycotoxins, genetics and behaviour, which remain purely speculations.

1.2. Infectious organisms, host and environment factors in the nursery

The working hypothesis in this thesis is that PFTS is an infectious disease. In an infectious disease, the causative organism is obviously a necessary cause, without which the disease would not occur. However, host and environmental factors, with differing weights on their contribution to causality, together with the organism bring about the disease. Together, they are the sufficient cause. The following section briefly reviews relevant aspects of organisms, host factors and environmental factors in nursery pigs and contribution to postweaning disease of nursery pigs. As this is a very broad topic, it is presented in the context of PFTS.

1.2.1. Overview of common nursery pathogens and PFTS-relevant pathogens

1.2.1.1. Porcine circovirus type 2 (PCV2)

PCV2 is an important pathogen that can cause postweaning anorexia and loss of body condition in pigs, and is a major differential diagnosis for PFTS. PCV2, which belongs to the family Circoviridae, is a circular single-stranded DNA virus that causes PMWS and other PCVAD. PCV2 is further divided into 3 genotypes, PCV2a, 2b and 2c, with only 2a and 2b associated with clinical diseases to date.⁸ The spectrum of clinical signs associated with PCV2 are wide, including anorexia, weight loss, icterus, pneumonia, diarrhea and reproduction failure.¹⁷⁷ Granulomatous inflammation of the lymphoid organs, lung and kidney, with or without

amphiphilic botryoid cytoplasmic inclusion bodies in macrophages is the hallmark of PCV2 infection.²⁹ Despite PCV2 being ubiquitous in commercial farms, not all farms or infected pigs develop clinical disease. Thus, to diagnose PCVAD, the presence of clinical signs, characteristic histological lesions and viral DNA or antigen within the lesion are all required.^{23,183} This makes neither conventional PCR nor quantitative real time PCR a preferred diagnostic tool for individual animals, but they are sensitive assays used to rule out PCV2 infection, not disease. That being said, pigs with PCVAD have higher serum loads of PCV2 than pigs without clinical disease.¹¹⁶ The vaccines currently marketed are effective. Although the commercial vaccines available globally are based on PCV2a strains, they are effective against both 2a and 2b diseases. It should be noted that the current vaccines reduce clinical disease and mortality effectively but do not protect against infection.⁸

1.2.1.2. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)

PRRSV, an Arteriviridae family member, is a positive-sense, single-stranded RNA virus. The highly diverse PRRSV is able to cause reproductive failure in sows and respiratory disease in suckling, nursery and older pigs. The nursery pig is an important target for clinical disease caused by PRRSV, because maternally derived antibodies wane during the nursery period, 4-8 weeks postweaning. Nursery pigs affected by PRRSV may demonstrate subclinical infection without sickness, or may develop clinical signs such as anorexia, lethargy and dyspnea.⁹¹ Gross lesions are not specific but non-collapsed lung and enlarged edematous lymph nodes warrant the inclusion of PRRS as a differential diagnosis in nursery pigs. Histological changes in PRRSV-affected nursery pigs may include combinations of the following: interstitial pneumonia with macrophage necrosis and infiltration, lymphoid depletion to hyperplasia depending on the stage of infection, vasculitis and myocardial necrosis, and non-suppurative encephalitis.¹⁶⁸

Prevention of the entry of PRRSV to the farm is crucial, because PRRSV typically persists in the farm once entered. Farms can be infected with multiple strains simultaneously. However, eradication of PRRSV from a herd with high economical value has been reported.^{34,36} Commercial vaccines are available, but satisfactory protection by vaccination is variable and not guaranteed.²¹¹

1.2.1.3. *E. coli*

E. coli is a cause of postweaning diarrhea. Enterotoxigenic *E. coli* (ETEC) strains that possess F4 and F18 fimbriae and one or more enterotoxins are most commonly associated with postweaning diarrhea, while the role of AIDA-bearing ETEC is less clear.⁴⁷ Enteropathogenic *E. coli* (EPEC) associated with attaching and effacing lesions may also be encountered in postweaning pigs with or without diarrhea. Although disease is most commonly observed in the first week postweaning, the use of prophylactic antibiotics can delay the onset of clinical signs by weeks.⁴⁸ Diarrhea and dehydration are the most common clinical signs, although acute death can occur. Diagnosis of ETEC can be achieved by observation of clinical signs and bacterial attachment to enterocytes without obvious cytopathic effect, isolation of characteristic beta-hemolytic bacteria and subsequent demonstration of fimbrial antigens and toxin genes.⁴⁷ The diagnosis of EPEC is more challenging, because EPEC strains are typically non-hemolytic. Thus, EPEC strains cannot be readily distinguished from the non-pathogenic strains on the basis of hemolysis, making the downstream testing for virulence factor less feasible.²¹¹ The logical steps to diagnose EPEC-associated diarrhea are the presence of clinical signs, the observation of characteristic attaching and effacing lesions, and then demonstration of EPEC strains by culture and subsequent tests.

1.2.1.4. Rotaviruses

Rotaviruses belong to the genus Rotavirus, family Reoviridae and are non-enveloped double-stranded RNA viruses. Four serogroups based on antigenicity of viral protein VP6 are reported for pigs: Group A, B, C and E.²⁴

Group A rotavirus is a well documented cause of neonatal diarrhea.^{14,24} Uncomplicated group A rotavirus infection causes mild diarrhea for 2 to 3 days. Degeneration of small intestinal villous epithelium and subsequent villous atrophy are the characteristic, but non-specific, histological changes. Morbidity of neonatal infection is typically less than 20%, within which only less than 15% (i.e. <3% total mortality) die from dehydration.²⁴ Although it can also be detected in postweaning pigs with diarrhea, the role of group A rotavirus in postweaning diarrhea is less clear. In a study by Janke, group A rotavirus was detected in 41% of nursery pigs with diarrhea.⁸⁷ Interestingly, during experimental inoculation of nursery pigs with pathogenic *E. coli*, it was demonstrated that pigs infected with one particular strain of pathogenic *E. coli* did not develop diarrhea unless fecal shedding of group A rotavirus was co-present, whereas pigs not infected with *E. coli* but shedding group A rotavirus did not develop diarrhea.¹²⁰ These findings suggest that group A rotavirus in nursery pigs is at least a co-pathogen of diarrhea but infection of nursery pigs may not lead to clinical disease.

Recent PCR evidence revealed that group B and C rotaviruses are also prevalent in suckling and nursery pigs.^{110,111} Single infections of group B and C rotaviruses were present, but mixed infections of two or three groups were most common. The virulence of group B and C rotaviruses in separate age groups of pigs, as well as their biological impact in the porcine industry await further characterization.

1.2.1.5. Coccidia

*Cystoisospora suis*¹⁷⁶ (previously *Isospora suis*) is probably the only coccidian parasite significant for the North American pig industry. The sporulated oocysts are highly resistant to most disinfectants.¹⁰⁶ Sows do not appear to be the major source of infection.¹⁰⁶ Recent research indicates that maternally derived antibodies do not protect piglets from infection.¹⁷⁶ *C. suis* mainly causes diarrhea in suckling pigs but can also affect nursery pigs. Increasing age is thought to be negatively associated with susceptibility to *C. suis* infection.²⁰⁸ Based on experimental inoculation of up to 2×10^6 oocysts per pig, pigs inoculated at 4 weeks and 6 weeks of age did not develop significant gross and histological changes.¹⁹⁰ Thus, the true impact of *C. suis* in the nursery is not known. Infective dose is also a factor that affects the clinical outcome of *C. suis* infection; larger doses induce more severe disease and pathology.¹⁹⁰ Following infection, affected pigs initially have diarrhea of pasty fecal consistency, that becomes more watery with time.¹⁰⁶ Demonstration of characteristic oocysts in clinically affected pigs by fecal flotation, intestinal smear and histology are all methods used to diagnose *C. suis* diarrhea.¹⁰⁶ Pathological changes include small intestinal villous atrophy and in more severe cases, necrotizing enteritis with intra-lesional organisms. Pathology appears initially in the jejunum and as infection progresses, the ileum becomes more severely affected.¹³¹ Totrazuril, which is effective for both sexual and asexual stages of *C. suis*, is an effective treatment.²¹¹ However, good management and sanitation to reduce exposure to oocysts is an essential component of control.

1.2.1.6. Haemagglutinating Encephalomyelitis Virus (HEV)

HEV is a Betacoronavirus, which belongs to the family Coronaviridae. It is believed that HEV is pathogenic to pigs younger than 4 weeks.¹⁷¹ The virus is widespread in the swine industry and

capable of causing two conditions, encephalomyelitis and wasting and vomiting disease, and the two conditions can overlap in one outbreak. Clinical signs occur in naïve pigs and include anorexia, vomiting, dehydration and central neurological signs such as paddling, muscle tremors, and hyperesthesia,¹²¹ however subclinical infection is common. Older pigs and piglets that suckle immunized sows are usually free from clinical diseases. However, a recent outbreak in Argentina suggests that clinical signs continued from the suckling phase as 29% of the nursery pigs from the affected farrowing site demonstrated wasting.¹⁶¹ Experimental inoculation of colostrum-deprived neonatal pigs revealed that nasal mucosa, tonsils, lungs, and small intestine were the sites of primary viral replication.⁶ Viremia was thought to be of little or no significance to the clinical disease. Non-suppurative encephalomyelitis and ganglioneuritis of the stomach wall, particularly in the pylorus, were the reported histological changes.^{6,161} A PCR assay designed to detect HEV RNA based on amplification of the spike-like protein gene¹⁷⁹ and immunohistochemistry successfully demonstrated viral RNA and antigen respectively, *in situ* in samples collected from the Argentina outbreak.¹⁶¹ It has been suggested that HEV may play a role in PFTS¹⁶⁹, however, solid evidence for this has yet to be presented.

1.2.1.7. Porcine calicivirus

Both norovirus and sapovirus, different genera within the Caliciviridae family, have been detected in pigs. They are enteric caliciviruses that differ from vesicular stomatitis virus. The prevalence of norovirus is 20% according to a study conducted in the USA, and were exclusively detected in asymptomatic finishing pigs.²⁰⁴ In contrast, the prevalence of sapovirus was higher (62% in pigs of all age groups) and most prevalent in nursery pigs (83%).²⁰⁴ Based on inoculation studies, it appears that norovirus is less pathogenic compared to wild type sapovirus.⁶¹ Small intestinal villous atrophy induced by experimental challenge is not

distinguishable from other viral enteritidis.⁶¹ In field situations, the impact of calicivirus in swine populations has yet to be demonstrated. Besides electron microscopy, PCR assays using either genus-specific primers or universal calicivirus primers have been described to detect porcine calicivirus.²⁰² In the farm in western Canada where PFTS was originally described, electron microscopy revealed the presence of calicivirus-like viral particles.⁵⁷ Further study of the relationship between calicivirus and PFTS is needed.

1.2.1.8. Porcine cytomegalovirus (PCMV)

PCMV of the Betaherpesvirinae subfamily is the cause of inclusion body rhinitis. It is thought to be ubiquitous in swine populations worldwide. PCMV infection is mostly self-limited and mortality occurs only in neonatal pigs following aberrant systemic infection. Subclinical infection or mild upper respiratory disease such as sneezing is most common.^{122,155} Gross lesions, including catarrhal rhinitis and those indicative of vascular damage including edema and petechiation in multiple organs are usually seen only in pigs less than 3 weeks of age.¹²² The most characteristic histological lesion is the presence of basophilic, large intranuclear inclusion bodies in the nasal glands, salivary glandular epithelium and lesser renal tubular epithelium. Dr. Steve Henry, (Abilene, Kansas) observed a high prevalence of suppurative rhinitis in the PFTS pigs associated with PCMV infection and proposed an association between PCMV in PFTS.⁶⁹ Further research is needed to test this hypothesis.

1.2.1.9. Other pathogens in the nursery

Pseudorabies virus that causes respiratory and neurological signs, *Streptococcus suis*, *Haemophilus parasuis*, *Actinobacillus suis* and *Salmonella* spp. that cause bacteremia and septicemia, *Mycoplasma hyopneumoniae* that causes enzootic pneumonia, transmissible

gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) that cause enteritis are also significant pathogens that can affect postweaning health. Diagnoses of these diseases can be readily established through the combination of the presence of clinical signs, characteristic pathological changes and adjunct tests.

1.2.2. Host factors affecting health at weaning

1.2.2.1. Immunity

Host immunity obviously plays an important role in infectious diseases. Unfortunately, pigs at weaning are vulnerable to infectious diseases largely due to the status of their passive and adaptive immune systems.

Neonatal pigs receive the majority of their immunoglobulin from colostrum. Maternal immunoglobulin is absorbed into the blood and is vital to protect neonatal pigs from systemic diseases such as septicemia. The half life of the maternal immunoglobulin in the pigs' circulation is about 12-14 days.¹⁶⁶ After gut closure at about 24 hours of life, pigs can no longer absorb entire immunoglobulin molecules, but the immunoglobulin in sow's mature milk continues to provide mucosal immunity at the gut level.¹⁷³ The relative proportion of IgA in milk increases and that of IgG decreases during lactation.⁹⁶ However, it is important to note that the total immunoglobulin concentration, as well as each individual immunoglobulin (IgG, IgA and IgM), decreases continuously.^{56,96} Inevitably, the passively acquired immunoglobulin levels in piglets decays to a low point at or after weaning.

The immune system of weaned pigs is more developed compared to that of a neonate, but it is not yet fully mature. The results of an investigation of the numbers of immunoglobulin-secreting

cells in various organs of 1-, 4-, 12- and 40-week old pigs demonstrated that numbers of immunoglobulin-secreting cells in most sites of the intestine gradually increased with age.¹⁰ However, in spleen, bone marrow, peripheral and mesenteric lymph nodes, IgG-secreting cell numbers dropped between 1 to 4 weeks¹⁰. Another study evaluating the dynamics of T-lymphocyte sub-populations in pigs from day 1 to 6 months of age found that at 4 weeks, T-lymphocyte sub-populations ($\gamma\delta$ TCR⁺, CD4⁺, CD4⁺CD8⁺, CD8⁺CD3⁺CD4⁻ $\gamma\delta$ TCR⁻) had not reached levels equivalent to adults.¹⁸⁷ The biological relevance of these results may not be immediately clear, but all point to the fact that the immune system of pigs is not yet mature at the time of weaning.

Thus, as a result of the waning of passive immunity and partially mature adaptive immune system, nursery pigs are vulnerable to infectious diseases.

1.2.2.2. Gut health

The effect of weaning on the gastrointestinal tract is another biological factor influencing the health of the nursery pigs.

It has been well demonstrated that after weaning, pigs have reduced small intestinal villous length and increased crypt depth compared to those at weaning.^{66,72,158} These morphological changes are consistent with increased enterocyte turnover. The villous height and crypt depth of weaned pigs do not return to the level at weaning even after 9 days.⁶⁶ Interestingly, villous height measured 5 days postweaning positively correlates to daily dry matter intake and weight gain.¹⁵⁸

Transient inflammation of the gastrointestinal tract occurs after weaning. It has been suggested that reduced feed intake contributes to this inflammation. During the period of postweaning

anorexia, small intestinal CD8⁺ T-cells and matrix metalloproteinase stromelysin increase but subsequently return to a normal level when feed intake resumes and intestinal morphology recovers.¹¹⁵ Further, proinflammatory cytokine (e.g. IL-1 β , IL-6 and TNF- α) expression spikes during the first 2 days after weaning and then returns to baseline levels.¹⁵¹

Small intestinal enzymatic activities are also altered after weaning. Affected enzymes that have been investigated include lactase, glucoamylase, dipeptidyl peptidase, aminopeptidase, aminopeptidase, γ -glutamyl transpeptidase and alkaline phosphatase.⁷² These alterations typically last for 3 to 5 days and then gradually recover.⁷²

Further, various factors including weaning age affect intestinal barrier function after weaning. Decreased transepithelial electrical resistance and increased serosal-to-mucosal [³H]mannitol fluxes occur in the intestines of pigs weaned at 19 days,^{126,127} but not in those weaned at 28 days.¹²⁶ Feed intake also affects intestinal barrier functions. Pigs with high feed intake did not develop increased mannitol flux while those with low intake did.²⁰⁶ Further, the degree of stress at weaning also plays a role in small intestinal barrier function deregulation. Pigs that experienced transportation stress before entering the nursery room developed increased mannitol flux but this was not the case when pigs did not experience transportation before weaning.²⁰⁶

The change of intestinal microbiota after weaning has been investigated by some researchers. Castillo studied the cecal content before and after weaning and found that *Lactobacilli* decreased and *Enterobacteria* increased in numbers which resulted in significant increased *Enterobacteria-to-Lactobacilli* ratio.²² However, Montagne reported both of the above increased after weaned 7 days.¹²⁹ On the other hand, Swords reported that after weaning, *Bacteroides* spp. markedly increased in colon.¹⁹¹ Although there is little doubt that after weaning, bacteria

communities in the intestine undergo drastic changes, the effect of these changes on nursery pig health has not been shown and needs further research.

1.2.3. Environmental factors affecting early weaning growth and health

1.2.3.1. Feed and swine health

Mycotoxin and ergot contamination are significant negative factors impacting swine feed quality. The most important mycotoxins for pigs are aflatoxins and ochratoxin (OTA) produced by *Aspergillus* spp., deoxynivalenol (DON), zearalenone and fumonisin produced by different *Fusarium* spp.. Ergot alkaloids are produced by *Cleviceps purpurea*. Molds that produce mycotoxins are divided into field and storage fungi.⁸⁵ Field fungi include *Fusarium* spp.; they grow and produce mycotoxins before harvest, but grow poorly in storage conditions. Storage fungi include *Aspergillus* spp. and *Penicillium* spp., which can produce mycotoxins even at low grain temperature and humidity.¹⁴⁵

Although *Aspergillus flavus* is regarded as a storage fungus, it can produce large amounts of aflatoxins even before harvest. Thus, grains are at risk of aflatoxin contamination both before and after harvest.²⁵ Drought and day-night temperature higher than 21°C favour high aflatoxins contamination in grains.^{25,207} Thus, it is conceivable that geographic areas characterized by the above conditions are more affected by aflatoxins (e.g. southern USA)^{25,207}, whereas colder areas like Canada are at lower risk.^{3,108} Aflatoxins mostly affect corn, peanuts, cottonseed and milo.¹⁴⁵ They can be several fold more concentrated in the dried distillers grains with solubles fraction. B1 toxin is the most prevalent and toxic among aflatoxins. Aflatoxins are hepatotoxic. Clinical signs depend on the amount of intake, and can vary from reduced growth (dietary level between 200-400 ppb) to hepatic damage (more than 400 ppb).¹⁴⁵ Suppression of cell mediated immunity

and phagocytic function has also been described.¹⁴⁵

OTA is a nephrotoxin that can cause necrosis of the renal proximal convoluted tubules and in chronic cases, interstitial fibrosis.¹⁴⁵ OTA production is favored at lower temperatures (12-25°C).¹⁴⁵ Thus, it is rare in the USA and more problematic in Europe.¹⁴⁵ OTA has been detected in Canadian feed, but the impact on the swine industry is likely low, if any.³ In a study conducted in western Canada that quantified mycotoxin levels from 106 feed samples suspected of compromising animal health, 5 samples contained OTA, but none were from swine feed.³ Clinical signs of OTA toxicity are those of renal failure, including polydipsia and polyuria. Diarrhea, anorexia and dehydration can also occur.¹⁴⁵ The severity of OTA nephrotoxicity is dose dependent. Dietary levels of 200 ppb can induce mild renal lesions, 1000 ppb can induce polydipsia, reduced growth and azotemia, and 4000 ppb can cause renal failure.¹⁴⁵ Immunosuppression and delayed response to vaccination make pigs prone to infectious diseases when exposed to OTA.¹⁴⁵

DON is a common contaminant of cereal grains (e.g. corn, wheat and barley) that is associated with feed refusal.^{108,145} The distribution of DON in grains is worldwide. It is not uncommon for swine feeds to contain low levels of DON.¹⁰⁸ Dietary levels of more than 1 ppm are clinically significant. There is a linear reduction in feed intake and weight gain from 2 to 8 ppm of exposure. When the level reaches 20 ppm, complete feed refusal and vomiting can occur.¹⁴⁵ The reported immunological effects of DON are not consistent among studies. For example, feeding similar levels of DON to pigs either increased¹⁵² or decreased¹⁷⁰ the immune response to tested antigens. Thus, the immunological effects of DON need to be further elucidated.

Zearalenone is produced in high moisture and can contaminate corn and wheat.^{25,209} Optimal

temperatures for Zearalenone production are 7-21°C.¹⁴⁵ Zearalenone competitively binds to estrogen receptors. In prepuberal gilts, 1-3 ppm dietary level can induce vulvovaginitis and rectal prolapse. In cycling sows and gilts, 3-10 ppm may cause retained corpora lutea, anestrus and pseudopregnancy.¹⁴⁵ The effect on piglets is less described, but estrogenic effects have been observed.¹⁴⁵

Fumonisin mainly affect corn and induce pulmonary and hepatic pathology via disruption of sphingolipid metabolism.¹²⁴ Fumonisin are stable during grain processing but do not appear to be a major problem in North America.²⁰⁹ More than 120 ppm of fumonisin in the diet causes acute pulmonary interlobular edema.¹⁴⁵ Acute toxicosis can also cause abortion, which is assumed to be the result of fetal anoxia. Subacute exposure to a lower dose (50-100 ppm) causes hepatitis characterized by increase serum hepatic enzymes, increase mitosis and apoptosis of the hepatocytes.¹⁴⁵

Ergot is a parasitic fungus that infects cereal grains (such as oat, wheat and rye) and grasses.¹⁴⁵ Ergot alkaloids produced by ergot suppress prolactin and causeagalactia of sows, which subsequently elevates piglet mortality because of starvation.¹⁴⁵ Vasoconstriction and endothelial damage also occur and lead to peripheral dry gangrene. It is recommended that ergot bodies should be less than 0.3% prevalent in cereal grains, and ergot alkaloids less than 100 ppb in the feed to avoid negative effects on pigs.¹⁴⁵

The prevention of mycotoxin contamination of swine feeds including nursery diets is very important for maintaining swine health. Different agents and processing techniques had been developed to inactivate mycotoxins in feed and grains.^{25,209} Efforts to control fungal growth before harvest are the most effective means to reduce the risk of mycotoxin toxicity.²⁵ For

example, simply irrigating crops to reduce drought stress was able to substantially reduce (78% reduction) *Aspergillus flavus* and hence aflatoxins.³⁸ Early sowing, early maturation and early harvesting of crops can reduce the time for fungus growth and thus reduce mycotoxin levels.^{25,107} If pre-harvest methods fail to protect crops from mycotoxin contamination, post-harvest means are available to reduce the mycotoxin. Moisture control is likely the most important way to reduced fungi growth and mycotoxin production in storage.²⁵ Pelleting can also reduce fungal growth because the process includes moisture removal. Mechanically screening the grains can be used to remove broken kernels and grain dust, which usually contains high levels of mycotoxins.¹⁶⁴ Ammonia and ozone can be employed to treat grains to detoxify mycotoxins.²⁵ Feed additives to absorb or degrade mycotoxins can be used to reduce the effect of mycotoxins. Absorbing agents such as bentonites, zeolites and hydrated sodium calcium aluminosilicates can bind mycotoxins (mostly aflatoxins, but also DON and zearalenone).^{25,145} Yeast and yeast cell wall components are able to bind a wide range of mycotoxins, such as DON and zearalenone.^{25,85,89} Some bacteria enzymes can degrade or alter the structures of mycotoxins (e.g. DON, aflatoxins and zearalenone).^{25,89} Several assays are available to evaluate the level of mycotoxin and ergot contamination in feed.¹⁴⁵

Antibiotics and feed additives Antibiotics are probably the most effective feed additive used to improve nursery growth and protect pigs from respiratory and enteric diseases.^{32,157} In fact, most nursery diets in North America are medicated.^{32,37,162} Several antibiotics are approved for in-feed use to prevent or treat enteric, respiratory and systemic diseases, as well as to promote growth.⁸⁴ Antibiotics in starter diets are able to improve average daily gain (ADG) by over 16%.³² Although some antibiotics have been used for decades in the swine industry, their growth promoting effect is not reduced.³² In-feed antibiotics also prevent morbidity and mortality of

young pigs.³² Tetracyclines were shown to be the most often used category of antibiotics in nursery starter diet in both Canada and the USA.^{37,162,167} Postweaning diarrhea is an important health concern for nursery pigs. In-feed antibiotics are effective for preventing and reducing the incidence and impact of postweaning diarrhea. This can be demonstrated by the increase of postweaning diarrhea after some European countries banned certain in-feed antimicrobials.⁷²

Concerns about the induction of antimicrobial resistance in bacteria that may be transmitted to humans have led to a large body of research conducted to explore alternatives to in-feed antibiotics to promote postweaning growth performance and health, and reduce postweaning diarrhea.^{94,156} It has been shown that a reduction of protein levels in feed without antimicrobials but with supplemental of essential amino acids did not reduce the growth performance of nursery pigs. This has been thought to be the most efficient way to counter the negative effect of removing antibiotics from feed.¹⁸⁶ Further, low protein diets supplemented with essential amino acids significantly reduced postweaning diarrhea in an enterotoxigenic *E. coli* challenge model.⁷⁰⁻⁷² High levels of ZnO (2500 ppm) have also been shown to reduce postweaning diarrhea and promote nursery growth.⁷⁰ However, high dietary ZnO is only beneficial in the early nursery phase and is recommended to be limited to about 3 weeks after weaning.⁸² Similarly, high dietary levels of copper also act as a growth promoter.⁸² The effects of spray-dried animal plasma has been extensively studied and reviewed.¹⁹⁷ It has been shown that spray-dried animal plasma, especially that of porcine origin, increases feed efficiency.¹⁹⁷ Concerns were raised whether or not the use of porcine plasma poses a risk for transmission of infectious diseases. Taking a ubiquitous virus such as PCV2 as an example, it has been shown that although commercial spray-dried porcine plasma contained a high level of PCV2 DNA, the spray-dried plasma protein was not infective when fed to pigs.¹⁸¹ Other additives, such as probiotics and

prebiotics have also been considered as health and growth promoters.⁹⁹ A probiotic is “a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that, exert beneficial health effects in this host”. A prebiotic is “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon”.¹⁷⁵ In swine, probiotic bacteria such as *Lactobacillus sobrius*, certain strains of *E. coli* and yeast have been shown to promote growth and counter postweaning diarrhea in some experiments.⁹⁹ However, the wide use of probiotics in the swine industry has not been endorsed because the effects had not been shown convincingly in field settings.⁸³ Similarly, prebiotics, such as lactose and inulin, have been reported to promote beneficial bacteria growth, but the growth and health benefit to nursery pigs has yet to be shown.⁹⁹

The presentation, ingredients and nutrients of the feed are also obviously important for the performance and health of the weaned pigs. Starter feed need to be highly digestible in order to encourage feed intake.¹⁴⁹ Crumble starter feed is preferred over pellet form.¹⁴⁹ The level of essential amino acid lysine is recommended to be 1.3% for pigs from weaning to 7 kg. The early weaning diet should not contain more than 25% soybean meal (weaned between 21-28 days),¹⁴⁹ which can cause transient hypersensitivity of the gut.¹⁰⁵ Spray-dried porcine plasma, whey powder, lactose, antibiotics and other growth-promoting additives are all beneficial in starter diet to ensure performance.¹⁴⁹ For older pigs, the diet composition can be simpler to accommodate cost efficiency.

1.2.3.2. Water quality

Water quality is a factor that potentially affects nursery pig performance and health. Microbial contamination is an important consideration of water quality of pig farms, and the quantity of coliform bacteria has been used to evaluate the degree of water pollution.²¹¹ It was proposed by the Environmental Protection Agency (1973) that water for livestock usage should not contain more than 1000/100 ml coliform bacteria. However, it is understandable that pigs in a farm environment are continuously exposed to much greater numbers of bacteria than indicated in this guideline. Further, it is doubtful that coliform bacteria are truly representative of the microbial contamination level of the drinking water. It can be granted that water contaminated with pathogenic bacteria poses greater health risks to swine than those contaminated with non-pathogenic bacteria. Surface water is of higher risk of being contaminated by microbes compared to municipal water, but it has been shown that nursery pigs drinking from surface water performed as well as those drinking from municipal water.¹³⁶ Water pH ranging from 6.5 to 8.5 is thought to be acceptable to swine.^{137,150} Alkaline water affects the efficiency of chlorination.¹⁵⁰ It had been shown that alteration of the pH of drinking water caused precipitation of medication in the water and thus reduced availability of the medication.³⁹ Total dissolved solids (TDS) is another important water quality parameter, and over 7000 mg total soluble salts per liter of water is considered unsafe for pigs. McLeese *et al.* showed that when water was not medicated, nursery pigs consuming high TDS (4390 mg/L) water had reduced weight gain compared to those consuming low TDS (217 mg/L) water.¹¹⁸ However, medicated water eliminated this effect.¹¹⁸ Nitrate and nitrite are important contaminants of water fed to livestock. The Canadian Water Quality Guidelines for livestock recommend maximum levels of water nitrate and nitrite to be 100 and 10 ppm, respectively.¹¹⁸ Water high in nitrate and nitrite can affect vitamin A utilization, reduce growth performance, and increase the prevalence of

diarrhea.^{137,150} The maximum sulfate content in swine drinking water is recommended to be 1000 ppm.¹⁵⁰ It was shown in a survey that 25% of the well water sources used in swine farms exceeded this limit.¹¹⁷ High sulfate levels in water can cause diarrhea in pigs.¹³⁷ However, it was demonstrated that nursery pigs could tolerate water sulfates of 1650 ppm without negative growth and health impacts.¹⁴⁸

1.2.3.3. Nursery management

Good management of the nursery room is vital for health of the pigs. Properly designed nursery pens need to provide adequate space for feeding, comfortable drinking, sleeping, and defecating areas for the pigs.^{21,211} Overcrowding facilitates the spread of infectious diseases because of the increased direct contact among animals. The Canadian National Farm Animal Care Council has proposed a new Code of Practice for The Care and Handling of Pigs (2013).¹ According to this code, if the heaviest nursery pig at the end of the nursery phase weighs 20 kg, the minimum space allowance is 0.25 m²/pig.¹ Enough feeder space can reduce competition during feeding and hence less stress for the pigs. Feeder space for each pig that weighs 4-23 kg was recommended to be 15-18 cm, and 30-35 cm for pigs over 23 kg.²¹¹ Not enough water consumption can reduce feed intake. Water access should not be limited and should be provided at two locations in each pen. For a nursery pig, water flow should be 0.5-1 L/min. Water nipple height is recommended to be 35 cm when the drinker angle is 45°, and 30 cm when 90°. ¹ Temperature of the nursery room is also important to the health of nursery pigs. Optimal temperature for newly weaned pigs is 27°C (desirable limits 24-30°C) which is gradually reduced to 21°C as pigs grow.^{1,211} However, the air temperature may not represent what the pig experiences (effective temperature). Air speed, type of flooring, humidity and group size, among others, can affect effective temperature.^{1,211} Nursery pigs should be protected from air speed over 0.25 m/s. ¹ In practice, the behaviour of the

pigs is good indicator of whether they feel comfortable with the temperature. A pig should sleep on his side exposing the abdomen. Chilled pigs will huddle together and pigs in heat stress pant.¹⁴⁹ The target minimum ventilation rate for 14-23 kg pigs is 0.0014–0.0019 m³/s-pig.²¹¹ High humidity also predisposes pigs to diseases such as *Streptococcus suis*.³³ The target relative humidity of the nursery room is between 40-70%.²¹¹ Proper levels of fresh feed in the trough is important to encourage newly weaned pigs to explore their new food source.⁵⁹ Regularly adding new feed to the feeder can trigger pigs to investigate the feeder.¹⁴⁹ Sanitation between batches of nursery pigs can greatly reduce disease transmission between batches and provide a clean environment for the new pigs. Washing and removing organic matter can remove 90% of the environmental bacteria, an additional 6 to 7% then can be killed by disinfectants, and a further 1-2% will be killed by fumigation.^{41,130} Infectious organisms can be protected from disinfectants in organic materials such as feces, feed and biofilms, thus removing the organic matter is prerequisite for effective disinfection. All the factors above should be considered together to ensure successful management of nursery pigs.

1.3. Methods of detecting infectious organisms

An inescapable part of establishing that a disease is of infectious etiology is to identify the causative organism. This section briefly reviews different methods to detect infectious organisms, in context of their applications in veterinary diagnostics and research when applicable.

1.3.1. Human eye and microscopy

The human eye is an innate instrument for pathogen detection, and occasions exist where our eyes can make a definite diagnosis. For example, *Haemonchus contortus*, a blood-sucking nematode found in the abomasum of small ruminants where it causes anemia and

hypoproteinemia, can be identified with the naked eye by the characteristic barber's pole appearance. With the help of light microscopy, many more organisms can be detected with a reasonable degree of certainty. The microscopic differentiation of parasitic eggs by fecal flotation falls into this category. Some fungal organisms, such as *Blastomyces dermatitidis* can be recognized in histological section by its broad-based budding in the background of pyogranulomatous inflammation. Histopathology can also reveal the presence of bacteria that can be categorized by morphology (e.g. rods, cocci, etc.), and can even give a hint of the presence of viral pathogens if inclusion bodies are observed. Electronic microscopy (EM) affords the human eye greater resolution. Except for prions, all microorganisms can theoretically be observed through EM. It should be noted that as magnification increases, the amount of sample that can be evaluated decreases accordingly. The naked eye can examine an entire animal, while EM evaluates tissue that is only millimeters wide.¹⁴⁶ Thus, selecting the correct sample(s) that contains the organism is important. This is also true for any other method that is used to evaluate tissue samples collected from one or more animals. Understandably, sampling from sites with significant lesions helps to enhance the chance of detecting the causative organisms.

1.3.2. Culture-based methods

Culture on either liquid or solid media is still one of the most important techniques in detecting and identifying bacteria. For example, for the diagnosis of Johnes' disease caused by *Mycobacterium avium* subspecies *paratuberculosis*, the widely used gold standard is bacterial culture, despite the existence of advanced molecular methods.¹⁹⁵ In pigs, different culture techniques had been developed for common pathogenic bacteria. For example, Modified Semisolid Rappaport Vassiliadis agar culture was found to be more sensitive, specific and accurate than a commercial PCR method for the detection of *Salmonella* spp..⁴⁴ Chocolate agar

or blood agar with adjacent *Staphylococcus aureus* nurse culture are used routinely to culture *Haemophilus parasuis*.¹⁴²

Similarly, the use of tissue culture to isolate viruses is useful in that it is relatively broad-spectrum (compared to sequence-based methods) and can detect and replicate live viruses, which can facilitate subsequent characterization of the virus in question. For example, PK15 cells (porcine kidney cell line) contributed to the first isolation of PCV2 from pigs with PCVAD.⁴³ Thus, virus isolation by tissue culture is still an important tool in investigating new diseases caused by putative novel viruses, although it is becoming a less frequently used diagnostic tool.

1.3.3. Immunological methods

Based on antigen-antibody reactions and various detection systems, immunological methods are widely used in veterinary research and diagnostics to detect either antibody to or antigen of an organism. Immunohistochemistry and fluorescent antibody techniques can demonstrate the presence of pathogens *in situ* and are part of the diagnostic criteria for PCVAD.²³ Enzyme-linked immunoassay (ELISA) is widely used to detect antibodies against pathogens and the throughput is reasonably high (i.e. tens of samples can be processed at the same time). Immunoperoxidase monolayer assay (IPMA) is a frequently used method to evaluate PCV2 titer.⁹⁸

Haemagglutination-inhibition (HI) may also be employed to detect antibodies for haemagglutinating viruses, such as swine influenza virus.⁹⁷

Recently, the development of fluorescent microsphere immunoassay (FMIA) greatly advanced immunological methods by its ability to simultaneously perform up to 100 tests. An FMIA assay has been developed to detect PRRSV antibodies in both serum and oral fluid.¹⁰¹ Similarly, a

multiplex FMIA for simultaneous detection of serum antibodies against PCV2, PRRSV and SIV has been recently developed by a research group from the Kansas State University.²⁰⁵

1.3.4. Methods to detect DNA or RNA sequences of infectious organisms

The development of PCR¹⁷² greatly advanced the ability of scientists to detect microorganisms. With knowledge of a limited length of the organism's genome, a pair of short oligonucleotide primers can be designed and the targeted sequence between the primers amplified enzymatically. The amplified products can be analyzed by electrophoresis with or without blotting. Further, this end-point analysis can be replaced by real-time detection of the products by either fluorescent dyes or probes. The real-time version of PCR not only saves time by eliminating the electrophoresis, it more importantly enables quantification of the target by the addition of standards with known target copy numbers. With a mixture of different sets of primers (and probes), both conventional and real-time PCR can be multiplexed.

Loop-mediated isothermal amplification (LAMP)¹³⁵ which operates under isothermal conditions and does not require a specific thermo cycler, makes on-site field detection of nucleic acid possible.

Hybridization-based techniques, on a macro- or micro- scale can not only confirm the presence of specific target sequences, but can also detect many microorganisms simultaneously. For example, the pan-viral microarray contains conserved sequences (probes) of all known viruses and offers wide spectrum screening of potential viruses in the sample.²⁷ This technique was used to discover Reston ebolavirus in pigs.⁷ Although the pan-viral microarray theoretically can detect all viruses, its applicability for the detection of novel viruses is limited by the similarities between the viral sequence and the sequence of the probes on the chip.

In situ hybridization is used to demonstrate the presence and location of DNA or RNA of a microorganism on a histological section. With improved signal demonstration techniques (thus increased analytical sensitivity),⁵ *in situ* hybridization is a powerful alternative to immunohistochemistry with the advantage that no development of specific antibodies is needed.

Methods to detect microorganisms that do not require knowledge of genetic information of the microorganisms are now available and their use is increasing. Representational difference analysis (RDA) of cDNA can detect differences in genetic material present in two samples (e.g. case vs. control),⁸⁰ and its use led to the discovery of a murine norovirus that caused hepatic necrosis in STAT1^{-/-} mice.⁹⁰ Other sequence-independent methods that were used before the “high-throughput sequencing” era, such as sequence-independent single-primer amplification and random PCR among others, have been reviewed recently.^{9,128}

Next-generation sequencing technologies, however, are probably the most powerful tools for the future of pathogen detection and discovery. Various high throughput sequencing platforms produce up to several orders of magnitude more sequence reads per sample compared to technologies based on Sanger sequencing.⁹⁵ Veterinary research has begun to utilize and benefit from these new technologies. The discovery of an astrovirus associated with shaking mink syndrome,¹³ the characterization of pig fecal virome,¹⁸⁰ establishment of causal relationship between proventricular dilatation disease of psittacine birds and avian borna virus,⁷⁵ all involved the application of high-throughput, next-generation sequencing.

Scientific methods are approaching the capability to sequence literally every nucleotide in a sample. The sequence data, combined with advanced bioinformatics analysis, can theoretically enable detection of every microorganism in a given sample. However, the problem becomes how

to understand the role of these microorganisms – i.e. how do we know whether these organism cause disease or not?

1.4. Establishment of disease causation with emphasis on infectious disease

Much of the work presented in this thesis is centered on the hypothesis that PFTS is an infectious disease. Determining the etiology of a disease, even with today's advanced technology, is not always an easy task. The kinds of evidence that can establish disease causation has been extensively discussed and reviewed, especially during the past one hundred years.^{35,46,50} The following section aims to provide some background useful for the interpretation of data collected in the PFTS investigation.

1.4.1. Henle-Koch Postulates

With the improved ability to discover microorganisms, the germ theory of disease gained popularity in the 19th century.⁵⁰ However, as larger and larger numbers of unique microorganisms were identified, investigators were faced with the need to differentiate between pathogenic and non-pathogenic (commensal) organisms. In this historical scene, Robert Koch (likely under Jacob Henle's influence) developed criteria to determine if a microorganism was the cause of a disease.¹⁶⁵ These criteria later became known as “(Henle-) Koch's postulates” and are as follows (based on Rivers' translation)¹⁶⁵:

"1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.

2. The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.

3. *After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.*”

If the above can be proved, “*then the occurrence of the parasite in the disease can no longer be accidental, but in this case no other relation between it and the disease except that the parasite is the cause of the disease can be considered.*” (Rivers’ translation ¹⁶⁵)

1.4.2. Difficulties in fulfilling Henle-Koch postulates

In Koch’s day, the causative agents of anthrax, tuberculosis, erysipelas, tetanus and most other infectious animal diseases fulfilled these postulates.⁴⁶ However, Koch himself and many others recognized that these criteria cannot be applied rigidly. Organisms that fulfill these criteria are almost undoubtedly the cause of the disease in question, but those that cannot are not necessarily excluded as the etiology of the disease in question. That is to say, the Henle-Koch postulates are sufficient but not necessary evidence for causation. Even neglecting the technical problems with fulfilling the first criterion (i.e. sensitivity of the detection method and timing of the sampling among others), the second and third criteria of the postulates can be difficult or impossible to fulfill.

Obviously, organisms that exhibit a subclinical carrier state or those that act as opportunistic pathogens will not satisfy the second criterion of the Henle-Koch’s postulates. In the context of swine diseases, PCV2 is an excellent example of this, in that it is considered to be nearly ubiquitous in swine populations but not all infected pigs develop PCV2-associated diseases (PCVAD).¹⁷⁷ Thus it was no surprise that the swine community was skeptical about its causal relationship to the clinical syndrome.¹⁷⁷ However, evidence that PCV2 is the pathogen that causes porcine postweaning multisystemic wasting syndrome (PMWS) (one of the PCVADs)

was very strong based on the presence of large amount of antigen in the characteristic lesions³⁰ and that vaccination effectively reduced mortality associated with PCV2 infection.⁸ Porcine Reproductive and Respiratory Syndrome (PRRS) is another pertinent example. Despite the claim in the early 1990s that PRRSV fulfilled the Henle-Koch postulates¹⁹³, subclinical infection of PRRSV is common especially when the virus is endemic.¹³⁴ In this sense, PRRSV actually cannot be said to have fulfilled the Henle-Koch's postulates; however, few doubt that it is the causative agent of PRRS.

The challenge to fulfill the third criterion of the Henle-Koch's postulates is two-fold, since it requires both isolating the organism in pure culture, and experimental reproduction of the disease in question using the isolated organism. The microorganisms that satisfied the postulates in the early days were bacteria that could be isolated using acellular culture media (the strictest sense of the original postulates). However, obligate parasitic organisms such as viruses and intracellular bacteria cannot be cultured in acellular media, thus, it was impossible to fulfill the postulates in a literal sense. Because of this, Rivers suggested conditions for establishing the causal relationship of a virus and a disease that did not require culturing the virus (even in modified tissue cultures) as a criterion.¹⁶⁵ In the 1990s, molecular techniques to detect viruses (and other organisms) were further proposed to replace the requirement for culturing.⁵⁰

Reproducing the disease experimentally is another problematic requirement to satisfy the Henle-Koch's postulates. PCV2 again demonstrates this challenge in that PCV2 inoculation alone, without manipulating the host immunity, injection of immune stimulants or co-infection with another organism, is seldom successful in reproducing PCVAD.¹⁹⁶

1.4.3. Modern guidelines for establishing infectious disease causation

The difficulties described above were indeed recognized by Koch himself.⁴⁶ Fredericks and Relman commented that the power of the Henle-Koch's postulates "*comes not from their rigid application but from the spirit of scientific rigor that they foster*".⁵⁰ Koch intended to convince skeptics of his day (1880's) that bacteria caused diseases such as tuberculosis by setting up very strict criteria.⁵⁰ After the germ theory of disease had been firmly established in the scientific community, new guidelines were proposed for establishing infectious disease causation. From the 1930s to 70s, different criteria for disease causation were proposed. Especially noteworthy are Hill's epidemiological criteria for causation, which are of specific relevance to the current thesis.⁷³ The first three of Hill's criteria speak to the association of a putative causal agent or event to the disease in question: the strength, consistency and specificity of the association.⁷³ Other proposed criteria within this period include Rivers's conditions for establishing the specific relationship of a virus to a disease¹⁶⁵, Evans's immunological proof of causation⁴⁵, and criteria for establishment of viral causation of cancer, and chronic diseases.⁸⁸ These emerged in the context of specific scientific discoveries of infectious agents and diseases. Evans attempted to take into account the common aspects of these later criteria and proposed ten criteria for causation⁴⁶ as follows:

- 1. Prevalence of the disease should be significantly higher in those exposed to the putative cause than in controls not so exposed.*
- 2. Exposure to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant.*
- 3. Incidence of the disease should be significantly higher in the exposed to the putative cause than in those not so exposed as shown in prospective studies.*

-
4. *Temporally, the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell shaped curve.*
 5. *A spectrum of host responses should follow exposure to the putative agent along a logical biologic gradient from mild to severe.*
 6. *A measurable host response following exposure to the putative cause should regularly appear in those lacking this before exposure (i.e., antibody, cancer cells) or should increase in magnitude if present before exposure; this pattern should not occur in persons not so exposed.*
 7. *Experimental reproduction of the disease should occur in higher incidence in animals or man appropriately exposed to the putative cause than in those not so exposed; this exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure.*
 8. *Elimination or modification of the putative cause or of the vector carrying it should decrease the incidence of the disease.*
 9. *Prevention or modification of the host's response on exposure to the putative cause should decrease or eliminate the disease.*
 10. *The whole thing should make biologic and epidemiologic sense.*

Development of these unified concepts was a milestone for scientists seeking to establish causation of diseases based on rigorous evidence and sound reasoning, and they are still largely suitable guidelines to use in present disease investigations. However, one factor not addressed by these guidelines pertains to quantities of the putative causative organism in diseased and

not-diseased groups. It is understandable that in Evans's time, quantitative techniques for pathogen detection were not yet well developed. Real-time PCR¹⁷², has dramatically increased scientists' ability to detect and quantify organisms in samples. Fredricks and Relman brought this scientific advancement into their molecular guidelines of infectious disease causality.⁵⁰ Many of Fredricks and Relman's guidelines were a reaffirmation of Evans's concepts in the context of sequence-based identification of organisms, but the quantity of an organism's genome was taken into consideration. They state in their second guideline: "*Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.*"⁵⁰ Another noteworthy guideline is that efforts should be made to demonstrate that the organisms reside within lesions (the sixth guideline).⁵⁰ Obviously, in the same animal(s), demonstrating the organism or organism's genome at the site of pathology and not in normal tissue is a powerful indication that the organism is likely causally related to the disease in question.

Application of both of the above two guidelines (i.e. quantity of the organism and location of the organism) was well demonstrated in the discovery of PCV2 and PMWS. Although PCV2 was thought to be ubiquitous, pigs with PMWS have greater quantity of the virus compared to subclinically infected pigs.¹¹⁶ As mentioned above, PCV2 was detected in large amounts within lesions³⁰ and this later became one of the diagnostic criteria for PMWS.²³ It is noteworthy in the context of affirming the causal relationship of PCV2 to PCVAD that, after commercial vaccines were demonstrated to control PCVAD, the ninth of Evan's criteria had also been fulfilled.

2. Rationale, hypotheses and objectives

The etiology of PFTS is not obvious based on the available literature. At the onset of this research, the only evidence that pointed to an infectious etiology was circumstantial evidence pertaining to the effect of desiccation of the farrowing and nursery rooms with aerosolized hydrated lime in one affected farm in Saskatchewan. On the other hand, there was no evidence to support that management, feed and water quality was associated with PFTS. Thus the author hypothesized that PFTS is an infectious disease.

The objectives of this thesis are:

- 1) To develop a clinical definition of PFTS.
- 2) To determine the pathologic lesions most characteristic of PFTS based on a case-control study of affected and non-affected pigs from affected farms.
- 3) To determine whether previously characterized porcine pathogens are associated with PFTS.
- 4) To determine if any specific serum chemistry is associated with PFTS, and whether this specific serum chemistry reflects pathology in a specific organ(s) or biological system.
- 5) To develop a snatch-farrowed porcine colostrum-deprived (SF-pCD) animal model that can be used for the experimental reproduction of PFTS.
- 6) To reproduce the clinical signs of PFTS, specifically debilitating weight loss and repetitive oral behaviour, in SF-pCD pigs by inoculation of tissue homogenates collected from affected pigs.

3. Clinical presentation, case definition, and diagnostic guidelines for porcine periweaning failure to thrive syndrome

This chapter represents the first step in the scientific investigation of PFTS. In addition to describing the clinical presentation of PFTS, a clinical case definition is proposed, which serves as a tool to identify allegedly affected farms in the investigation.

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Huang Y, Henry S, Friendship R, Schwartz K and Harding J. Clinical presentation, case definition, and diagnostic guidelines for porcine periweaning failure to thrive syndrome. *J Swine Health Prod.* 2011;19(6):340-344

All authors contributed to proposal of the PFTS clinical definition and writing of the manuscript.

Porcine periweaning failure to thrive syndrome (PFTS) presents clinically as moderate but variable morbidity (range from 1% to 20%) with high case fatality. Individual pigs, affected within 7 days after weaning, demonstrate anorexia, lethargy, and progressive debilitation. PFTS has specifically been noted in proceedings and publications from veterinarians and researchers since 2008,^{42,57,200} although clinicians have observed weanling pigs with similar clinical signs for many years. In some farms, the clinical signs of PFTS may have been confused with or masked by other diseases, including porcine circovirus associated diseases (PCVAD), swine influenza, and porcine reproductive and respiratory syndrome (PRRS). The recognition of cachectic and debilitated pigs shortly after weaning that were PRRS virus (PRRSV) and swine influenza virus- (SIV-) negative and porcine circovirus type 2-immunized, suggested that PFTS is a distinct clinical entity. The authors are aware of PFTS or clinically similar cases reported in a number of different regions of North America. Little is known about the cause of PFTS, but infectious agent(s) and management-related factors need to be ruled out. In this paper, we propose a clinical case definition for PFTS, describe the characteristic clinical progression and signs and observed lesions, and make recommendations for the investigation of PFTS-suspected farms.

3.1. Obtaining the consensus name “porcine periweaning failure to thrive syndrome (PFTS)”

PFTS was previously reported as postweaning catabolic syndrome,⁴² postweaning wasting/catabolic syndrome,^{51,64} and failure to thrive syndrome,²⁰⁰ and is possibly the same disease as postweaning fading pig/anorexia syndrome.¹⁶⁹ At the 2010 International Pig Veterinary Society (IPVS) Congress in Vancouver, a number of researchers, diagnosticians, and practitioners from North America met and reached consensus that the name “periweaning failure to thrive syndrome (PFTS)” reflects the age of onset and clinical presentation of the syndrome. The authors encourage the adoption of this name when describing cases meeting the case

definition outlined below, until such time that new knowledge of the etiology and pathogenesis supports a more specific disease designation. We recommend that “periweaning” is preferable to “postweaning”, because there may be management or infectious factors both pre- and postweaning that contribute to the development or risk of PFTS.

3.2. Clinical case definition of PFTS

A proposed case definition for PFTS, revised and rephrased from Friendship et al,⁵¹ is as follows: “PFTS is characterized clinically by the progressive debilitation of weanling (nursery) pigs in the absence of discernible and detrimental infectious, nutritional, managerial, or environmental factors that can explain the clinical syndrome. At weaning, affected pigs are of average to above average body weight, and neither affected pigs nor their cohorts show evidence of residual illnesses from the suckling phase. Within 7 days of weaning, affected pigs are anorexic and lethargic. They deteriorate and within 2 to 3 weeks of weaning demonstrate marked muscle weakness and loss of body condition. Some affected pigs in all affected farms show repetitive oral behavior such as licking, chewing, or chomping. In affected farms, morbidity and mortality by batch varies over time, but case fatality is high.”

3.3. Clinical signs

The most frequently observed clinical signs of PFTS are listed in Table 3.1. It is important to emphasize that affected pigs and their cohorts demonstrate no obvious detrimental clinical disease at the herd level prior to weaning. At weaning, the pigs that will become affected appear healthy and of average to above average body weight and condition, and cannot be identified as “at risk.” Four to 5 days after weaning, the affected pigs have hollow abdomens or flanks, presumably the result of anorexia. Affected pigs appear hydrated. Frequent sneezing may be

heard in the nursery, but coughing and dyspnea are not typically observed. In affected pigs, abnormal oral behavior of repetitive licking or chewing motions, or a repetitive and intentional “chomping” activity, during which their heads may be drooped or elevated by resting the jaw on the back of a penmate may be observed, provided the observer remains motionless and quiet for a period of time. Such pigs are easily disturbed, making observation difficult. While identifying PFTS-affected pigs within the first week after weaning requires careful observation, affected pigs are easily identified by their lethargy, hollow abdomens, and failure to grow (Figure 3.1) by the second week post weaning. With quiet observation, affected pigs may be observed grouped together in a side-by-side stance with heads drooped. While this unusual posture may be maintained for long periods of time, pigs eventually become weak, lie down together, and may pile as if chilled. By 3 weeks postweaning, most of the affected pigs are severely debilitated (Figure 3.1), have died, or have been euthanized.

Most management interventions, environmental manipulations, and medical treatments are ineffective. That said, a critical review of all production practices must be performed to reduce stress, to prevent the exacerbation of all nursery diseases including PFTS, and to ensure animal welfare is maintained. Techniques that are consistently effective in reducing PFTS morbidity and mortality have not been accomplished.

3.4. Relevant gross and histological observations

The definition of PFTS remains currently at the clinical level, but the frequently observed gross and histological changes found in PFTS-affected pigs are summarized in Table 3.1. Whether or not these lesions are significant to the pathogenesis of PFTS needs to be determined. Necropsy examination reveals scant ingesta within the gastrointestinal tract. The small intestines of

affected pigs are empty or sometimes fluid-filled. The colon is empty or may contain pasty to liquid content. At later stages in the disease process, when most sick pigs are typically submitted for diagnostics, affected pigs have marked thymic atrophy, which is most obvious in the thorax. If affected pigs in earlier stages are examined, thymic atrophy appears to be less severe grossly, suggesting that it may be associated with prolonged anorexia or sickness, rather than a primary lesion. Bronchopneumonia may be observed in some pigs, especially those in later disease stages but is not a consistent finding. When the head is sectioned on the midline suppurative rhinitis, characterized by purulent material in the nasal cavity as proximal as the ethmoid conchae, is frequently observed in affected pigs as well as in some healthy penmates.

Microscopic lesions noted in PFTS-affected pigs include lymphocytic and suppurative rhinitis with or without cytomegaloviral inclusion bodies, lymphocytic superficial fundic gastritis, atrophic enteritis, and superficial colitis. Although the reasons for and the significance of these particular lesions are not clear at this time, they are prevalent in affected pigs and serve as pathologic indicators of PFTS. It is noteworthy however, that lymphocytic and suppurative rhinitis with or without cytomegaloviral inclusion bodies and superficial colitis have also been observed in healthy age-matched penmates of the affected pigs.⁷⁶

3.5. Recommendations for herd investigations

As the etiology and pathogenesis of PFTS are unknown, the presence of typical clinical progression, the age of onset, and the elimination by thorough diagnostic investigation of all other known porcine diseases as primary entities should be used to classify affected farms as having PFTS. A non-exclusive list of potential differential diagnoses based on clinical signs and pathology are listed in Table 3.2.

Recognizing PFTS-affected pigs in the early stage (ie, 4 to 5 days post weaning) is difficult but is vital to the proper interpretation of uncomplicated gross and microscopic pathology. Pigs that are sick for a prolonged period of time are at greater risk of succumbing to secondary infections which mask the original pathology. In our experience, an encompassing list of tissues must be evaluated microscopically (Table 3.3), even if they look grossly normal. Failing to collect certain tissues in suspected cases prevents the effective comparison with other cases, which is critical when characterizing a new syndrome such as PFTS. It is important to examine nasal turbinate, fundic stomach, small intestines, large intestine, and thymus histologically but these should not be the only organs examined. In addition, examining age-matched healthy cohorts from suspect farms is necessary to correctly interpret the significance of some gross and histologic lesions observed in sick pigs. For example, if specific lesions are of equivocal prevalence and severity in both sick pigs and healthy cohorts, the significance of these lesions is less certain. Special arrangements with the diagnostic laboratory are strongly recommended to reduce the substantial diagnostic costs associated with the examination of an extensive tissue set and the number of animals required in a disease investigation compared to routine diagnostic submissions. In our investigations of PFTS, it has also been useful to examine healthy pigs from unaffected farms in order to characterize the morphology of normal, healthy age-matched pigs.

Recommendations for the investigation of herds demonstrating signs typical of PFTS are listed in Table 3.4, which can serve as a checklist of actions to perform during investigation.

3.6. Implications

- Porcine PFTS is characterized by anorexia and lethargy of nursery pigs beginning soon after weaning, and is not associated with porcine circovirus type 2 , PRRSV, or SIV infection.
- The etiology and pathogenesis are unknown.
- The age of disease onset, presence of characteristic clinical signs, and elimination by thorough diagnostic investigation of other known porcine diseases as primary entities should be used to identify farms affected by PFTS.
- A thorough history, clinical examination, clinical pathology, gross and microscopic evaluation of affected pigs is necessary to work up suspected cases of PFTS. The pathologic examination should target affected pigs and age-matched healthy cohorts between weaning and 3 weeks post weaning.

Continued collaboration amongst clinicians, producers and diagnosticians is needed to identify cause(s), risk factors and prevention strategies for this syndrome.



Figure 3.1: Periweaning failure to thrive syndrome in nursery pigs. A: an affected pig showing a hollow abdomen; B: a severely debilitated pig.

Table 3.1. Most frequently observed clinical signs and pathological changes of periweaning failure to thrive syndrome

Clinical signs	Pathological changes
Anorexia	Chronic active rhinitis*
Lethargy	Lymphocytic superficial fundic gastritis
Standing but unwilling to move	Atrophic enteritis
Sneezing	Superficial colitis*
Repetitive licking, chewing, or chomping behavior	Thymic atrophy

* Lesions that have also been observed in age-matched cohorts.

Table 3.2. Potential differential diagnoses based on clinical signs and pathological changes associated with pigs affected with periweaning failure to thrive syndrome*

Signs and lesions	Pathogens
Debilitation, loss of condition	PCV2, PRRSV, HEV
Thymic atrophy	PCV2, PRRSV
Suppurative rhinitis	PCMV, toxigenic <i>Pasteurella multocida</i> strains
Gastritis	Helicobacter-like organisms, TGEV, rotavirus
Enteritis	Pathogenic <i>Escherichia coli</i> , <i>Salmonella enterica</i> serovars, TGEV, rotavirus, protozoans (eg, <i>Isospora suis</i>)
Colitis	Pathogenic <i>E coli</i> , <i>Salmonella enterica</i> serovars, <i>Brachyspira hyodysenteriae</i> , <i>Brachyspira pilosicoli</i>

* This is not intended to be a complete list of differential diagnoses. Swine diseases and pathogens exotic to Canada and the United States are excluded.

PCV2 = porcine circovirus 2; PRRSV = porcine reproductive and respiratory syndrome virus; HEV = haemagglutinating encephalomyelitis virus; PCMV = porcine cytomegalovirus; TGEV = transmissible gastroenteritis virus.

Table 3.3. Tissues to collect from pigs suspected of having periweaning failure to thrive syndrome*

Tissues with prominent lesions in affected pigs	
Tissues	Lesions in affected pigs
Gastric fundus	Superficial lymphocytic gastritis
Duodenum, jejunum and ileum	Atrophic enteritis
Spiral colon	Superficial colitis
Nasal turbinate	Chronic active rhinitis with or without cytomegalovirus inclusion bodies
Thoracic thymus	Thymic atrophy (grossly and histologically)
Tissues to rule out other potential pathogens or lesions†	
Tissues	Pathogens or diseases to rule out
Lung	PRRSV, SIV, PCV2, <i>Haemophilus hyopneumoniae</i> , <i>Haemophilus parasuis</i> , etc
Lymph nodes, tonsil and spleen	PCV2, septicemia, etc
Kidney	PCV2, glomerulonephritis, etc
Whole or half brain	<i>Streptococcus suis</i> , <i>H parasuis</i> , nonsuppurative encephalitis, etc
Pharynx and esophagus	Lesions that can compromise the passage of solid food
Gastric pars oesophagea	Ulceration and stenosis of the cardiac opening
Gastric pylorus	HEV-associated ganglionitis ⁸
Other tissues to collect for evaluation	
Tissues	Purpose of collecting
Liver, heart, pancreas, skeletal muscles	Rule out significant lesions; potential diagnostic use (eg, virus isolation, bacterial culture, PCR); maintain a thorough postmortem examination

* The tissues listed in this table are not exhaustive. Other tissues may be collected if deemed to provide useful information.

† The differential diagnoses in this table are examples and are by no means complete.

PRRSV = porcine reproductive and respiratory syndrome virus; SIV = swine influenza virus;

PCV2 = porcine circovirus type 2; HEV = haemagglutinating encephalitis virus; PCR = polymerase chain reaction

Table 3.4. Ten recommendations for the investigation of farms demonstrating signs typical of PFTS

1. Observe each production stage to identify sick pigs or clinical signs indicative of known infectious and noninfectious diseases, and perform diagnostics to investigate these as appropriate.
 2. Perform a site visit to audit and verify environmental conditions, management practices, feed and water quality and availability, and feeding practices.
 3. Obtain historical and current records of the morbidity and mortality of suckling, nursery and grower-finisher pigs. Verify temporal trends and confirm mortality levels in the nursery are elevated.
 4. Collect and analyze water and feed samples to rule out toxicities (eg, mycotoxins, herbicides, pesticides, etc) and potential nutrient imbalances, and to verify nutrient content.
 5. Observe nursery pigs when they are in a quiet state. This will allow the recognition of clinical signs that are hard to identify, such as “chomping.” Systematically observe nursery pigs of each week, starting with the youngest. Also carefully observe the oldest suckling pigs.
 6. Take multiple digital images and videos in different production areas of the farm, especially in the affected rooms. Look for and capture unusual; oral behaviour such as licking or chomping.
 7. Submit live pigs for thorough work-up including necropsy, histopathology and adjunct tests on multiple animals including sick and healthy age-matched cohort pigs.
 8. Whenever possible, identify pigs in the early disease stages for collecting diagnostic samples. However, it may also be useful to submit pigs of sequential ages and disease chronicities.
 9. Collect internal organs and other tissues for histology (Table 3), even if they lack visible gross lesions.
 10. Store tissues and sera at proper and varied conditions (chilled, -80° Celsius freezer, formalin-fixed, paraffin-embedded, RNA stabilizer, etc) to facilitate different diagnostic applications.
-

4. Diagnostic investigation of porcine periweaning failure-to-thrive syndrome in a farm from Saskatchewan: lack of compelling evidence linking to common porcine pathogens

This chapter presents a diagnostic investigation performed in a farm in Saskatchewan and was the first PFTS investigation undertaken. Several highly prevalent histological changes were found in this farm. None of the tested pathogens were clearly associated with PFTS pigs in this farm.

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Huang, Y., H. Gauvreau, and J. Harding, Diagnostic investigation of porcine periweaning failure-to-thrive syndrome lack of compelling evidence linking to common porcine pathogens. *J Vet Diagn Invest*, 2012. 24(1): p. 96-106.

Huang and Harding were responsible for the experimental design and necropsies. Huang contributed to the histological evaluation of tissues. Gauvreau was the veterinarian of the farm and contributed intellectually of the discussion of potential etiologies of PFTS.

4.1. Introduction

“Failure-to-thrive” is a term in the pork industry that refers to pigs that appear not to eat after weaning, and subsequently lose body condition and die with no other known etiology.

Importantly, failure-to-thrive pigs are not lightweight pigs (i.e., runts) at birth or weaning.

Commonly referred to as “starve-outs” by producers, such pigs have been observed at a low prevalence (up to 0.5% of total weaned) for many years. In the past, the condition received little

attention since the prevalence and economic loss was low. However, herd outbreaks of

failure-to-thrive pigs, resulting in nursery mortality that has risen well above historical baseline

levels, were recently recognized in a number of North American farms.^{42,57} The condition was

recently named “periweaning failure-to-thrive syndrome” (PFTS), and a clinical case definition

proposed as follows: “PFTS is characterized clinically by the progressive debilitation of

weanling (nursery) pigs in the absence of discernible and detrimental infectious, nutritional,

managerial, or environmental factors that can explain the clinical syndrome. At weaning,

affected pigs are of average to above average body weight, and neither affected pigs nor their

cohorts show evidence of residual illnesses from the suckling phase. Within 7 days of weaning,

affected pigs are anorexic and lethargic. They deteriorate and within 2–3 weeks of weaning

demonstrate marked muscle weakness and loss of body condition. Some affected pigs in all

affected farms show repetitive oral behavior such as licking, chewing or chomping. In affected

farms, batch morbidity and mortality varies over time, but case fatality is high”.⁷⁹ The etiology of

PFTS is presently unknown and may include infectious agent(s), noninfectious factors, or both.

A 100-sow farrow-to-finish farm has experienced disease causing fluctuating nursery mortality

beginning early 2007. The clinical presentation of this disease meets the definition of PFTS

quoted above. This farm is historically free of *Mycoplasma hyopneumoniae* and *Porcine*

reproductive and respiratory syndrome virus (PRRSV). Nursery mortality was elevated by 3.7 fold (7.2% for 2007–2009; 1.9% for 2004–2006), mostly attributable to failure-to-thrive piglets. Chomping behavior was observed in the failure-to-thrive pigs in this farm. Common mycotoxins (tested by the Veterinary Diagnostic Laboratory of North Dakota State University, Fargo, North Dakota) and herbicides (tested by ALS Laboratory Group, Saskatoon, Saskatchewan, Canada) in feed and water, respectively, were negative. Various managemental and medical interventions to control PFTS had been attempted, but failed to prevent mortality. The interventions included ventilation adjustments to prevent piglet chilling, changes in starter feed and water source, antibiotic treatments, H3N2 swine influenza vaccination of the breeding herd, and *Porcine circovirus-2* (PCV-2) vaccination of piglets at weaning. Although thorough desiccation of the farrowing and nursery rooms with aerosolized hydrated lime appeared to be beneficial during periods of elevated PFTS mortality, the cyclical nature of clinical signs and mortality on the farm made it difficult to evaluate the true impact of this procedure. On the other hand, it supports the hypothesis that infectious agent(s) may be involved in pathogenesis of PFTS. The objective of the current study is to report the diagnostic investigation of PFTS on this farm with emphasis on describing the most prevalent lesions and identifying the infectious agents in PFTS-affected compared to age-matched, nonaffected pigs.

4.2. Materials and methods

4.2.1. Sample collection

During periods of peak PFTS mortality, sick pigs with characteristic clinical signs (PFTS-SICK; $n = 18$), and healthy age-matched cohort pigs selected from the same weaning group and air space (PFTS-HLTHY; $n = 7$), ranging in age from 3 to 6 weeks old (1–3 weeks postweaning),

were selected on the farm. Although chomping behavior was observed on the farm during outbreaks of PFTS, the chomping status of the pigs included in the current study was unknown as the authors failed to recognize the biological importance of this clinical sign at the onset of the investigation. Four age-matched healthy pigs from each of 2 unaffected farrow-to-finish farms were handled similarly on separate occasions and included as a comparative group in the investigation (CTRL; $n = 8$). PFTS-SICK and PFTS-HLTHY pigs were sedated with azaperone (Merial Canada Inc., Baie d'Urfé, Quebec, Canada) (2.2 mg/kg intramuscularly) on the farm, and transported to the necropsy facility at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada). The CTRL pigs were transported unsedated because the length of time in transit was shorter, thus aggression during transport was less of a concern. Necropsy examinations were performed immediately after humane euthanasia using intravenous barbiturate overdose. An extensive list of tissues (Table 4.1) were collected and processed routinely for bacterial culture, histology, and adjunct testing for common swine pathogens. Fecal samples were collected from a subset of PFTS-SICK ($n = 5$) and PFTS-HLTHY ($n = 3$) pigs at the time of necropsy. Fecal samples ($n = 5$) were collected from age-matched pigs from 1 CTRL farm but on a different day and not from the necropsied pigs.

4.2.2. Diagnostic tests

Adjunct diagnostic tests were performed on appropriate tissues and samples (Table 4.2) using published or in-house protocols. The primers and conditions of polymerase chain reaction (PCR) testing are summarized in Table 4.3. Immunohistochemistry (IHC) was performed using a streptavidin–biotin complex technique adapted for an automated slide stainer (Fisher Scientific Co., Edmonton, AB, Canada) as previously described.⁸ The primary antibodies used for IHC in the present study are summarized in Table 4.4. Binding of the primary antibodies was detected

using biotinylated goat anti-rabbit immunoglobulin (Ig)G and horse anti-mouse IgG, (Vector Laboratories Inc., Burlingame, CA) and an avidin–biotin immunoperoxidase complex reagent, with 3,3'-diaminobenzidine tetrahydrochloride (Electron Microscopy Science, Ft. Washington, PA) as the chromogen.

Routine aerobic and anaerobic bacterial cultures were performed on the jejunum, colon, and lung. When hemolytic *Escherichia coli* was cultured, an agglutination test was applied to detect the presence of F4 fimbria.

The following pathogens were tested by either PCR or IHC (Table 4.2): PCV-2 (IHC, on lymphoid organs, stomach, small and large intestine), PRRSV (PCR, on lung), *Influenza A virus* (FLUAV; PCR, on lung), *Alphacoronavirus 1* (ACoV-1; previously known as *Transmissible gastroenteritis virus*; IHC, on stomach, small and large intestine), *Rotavirus A* (RV-A; IHC, on stomach, small and large intestine), Porcine enteric calicivirus (PECV; PCR, on small and large intestine), *Betacoronavirus 1* (BCoV-1; previously known as *Haemagglutinating encephalomyelitis virus*; PCR, on tonsil, lung, kidney, small and large intestine), *Suid herpesvirus 2* (SuHV-2; previously known as *Porcine cytomegalovirus*; PCR, on tonsil, lung, kidney, small and large intestine), *Torque teno virus 1* and 2 (TTV-1, -2; PCR, on spleen or bone marrow), *E. coli* virulence factors (AEEC, AIDA, Sta, STb, LT, SLT; PCR, on cultured colonies from small and large intestine), *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* (PCR, on large intestine). Except for SuHV-2, BCoV-1, and PECV, the above tests were performed by a commercial diagnostic service (Prairie Diagnostic Services Inc., Saskatoon, Saskatchewan, Canada). The PCR detection of SuHV-2, BCoV-1, and PECV was performed in-house according to published methods with minor modifications (Table 4.3).^{63,179,203} Fecal flotation using Wisconsin double centrifuge technique³¹ was performed to detect parasitic oocysts in the feces.

4.2.3. Statistical analysis

The frequency of gross and histologic lesions and pathogens in PFTS-SICK, PFTS-HLTHY, and CTRL pigs were compared using a Fisher exact test. Group differences were statistically significant if $P < 0.05$.

4.3. Results

4.3.1. Gross and histological lesions

On gross examination, all of the PFTS-SICK pigs were emaciated with loss of body fat reserves. The thoracic thymus was severely atrophic and sometimes barely visible. Gastrointestinal (GI) tracts were empty, and often much smaller in diameter compared to PFTS-HLTHY and CTRL pigs. Seven of 18 (39%) PFTS-SICK pigs had mild to severe cranioventral consolidation in the lungs. No gross lesions were observed in the PFTS-HLTHY and CTRL pigs (Table 4.5).

The most frequent histological lesions in PFTS-SICK pigs were thymic atrophy, superficial lymphoplasmacytic fundic gastritis, villous atrophy of the small intestine (duodenum, jejunum, and ileum), superficial colitis, lymphocytic and neutrophilic rhinitis, and mild nonsuppurative meningoencephalitis (Table 4.5). The frequency of these lesions was significantly higher in the PFTS-SICK than CTRL pigs (Table 4.5). In the thymus, the thickness of the cortex was severely reduced, yet the remnant of the medulla was still visible. In the stomach, the surface lining epithelial cells of the fundus were multifocally to diffusely attenuated, and mucus was lost in the cytoplasm. The glandular epithelial cells were occasionally necrotic. Moderate numbers of lymphocytes and plasma cells infiltrated the subepithelial lamina propria of the fundic stomach (Fig. 4.1). In fundus, no ganglionitis was observed. Unfortunately, pylorus was not examined in

the current study, where ganglionitis, if present, will be more likely found. The small intestinal villi were moderately to severely shortened (Fig. 4.2) and covered by low cuboidal to occasionally squamous epithelial cells. Low to moderate numbers of different stages of coccidia were present in the small intestinal epithelium of 6 out of 18 (33.3%) PFST-SICK pigs (Table 4.2). The colonic epithelium was disorganized, hyperplastic, or attenuated. Increased numbers of lymphocytes and plasma cells were present in the colonic lamina propria (Fig. 4.3). In approximately half of the PFST-SICK pigs, coccobacilli were observed attaching to the colonic epithelium, leading to rounding, necrosis, and sloughing of the epithelial cells, morphologically consistent with attaching and effacing *E. coli* (AEEC) infection (Table 4.2). The nasal mucosa was infiltrated by moderate to large numbers of lymphocytes, plasma cells, and neutrophils. The epithelial cells of the submucosal glands were frequently necrotic and attenuated. Large basophilic intranuclear inclusion bodies were occasionally observed in the glandular epithelium in a small portion of PFTS-SICK pigs, consistent with SuHV-2 infection, known commonly as inclusion body rhinitis (Table 4.2, Fig. 4.4). Mild nonsuppurative meningoencephalitis, characterized by lymphocytic to histiocytic perivascular cuffing around the meningeal and parenchymal vessels (Fig. 4.5), was observed in 6 of the 13 PFTS-SICK pigs in which the brain was examined histologically. The frequency of nonsuppurative meningoencephalitis in PFTS-SICK pigs was significantly higher than in PFTS-HLTHY and CTRL pigs.

Occasionally, other lesions were observed in the PFTS-SICK pigs, including various degrees of suppurative bronchopneumonia, lymphocytic nephritis, and fatty liver (Table 4.5). In the lung, bronchopneumonia was characterized by infiltration of neutrophils and consolidation of the parenchyma. Necrotizing bronchiolitis was not observed. The prevalence of bronchopneumonia in PFTS-SICK pigs was significantly higher than that in PFTS-HLTHY and CTRL pigs. No other

significant lesions were observed in the tissues sampled.

Rhinitis was present in all PFTS-HLTHY pigs and was (16.7%) or was not (83.3%) associated with cytomegalovirus inclusion bodies (Table 4.5). Atrophic duodenitis (85.7%), jejunitis (14.3%), and superficial colitis (71.4%) were also present in the PFTS-HLTHY pigs (Table 5). The prevalence of rhinitis, atrophic duodenitis, and superficial colitis in PFTS-HLTHY pigs was significantly higher compared to CTRL, but not different from PFTS-SICK pigs (Table 4.5). One CTRL pig had mild rhinitis and 1 had colitis. No coccidia were observed in the intestines of PFTS-HLTHY pigs, but 1 single macrogamont was detected in 1 out of 8 (12.5%) CTRL pigs. No other histological lesions were observed in any PFTS-SICK or CTRL pigs.

4.3.2. Detection of bacteria

No biologically relevant bacteria were consistently cultured from PFTS-SICK pigs (Table 4.2). No F4-positive *E. coli* or *Salmonella* spp., or target DNA of *B. hyodysenteriae* and *B. pilosicoli* were detected from any pigs. *Clostridium perfringens* was cultured from the intestines of some PFTS-SICK (22%) and PFTS-HLTHY (29%) pigs, but not CTRL pigs. Two *C. perfringens* isolates from PFTS-SICK pigs were tested by PCR for toxin genes (alpha, beta1, beta2, and epsilon toxin genes). Both isolates were only positive for alpha toxin and classified as *C. perfringens* type A (data not shown). *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis*, *Bordetella bronchiseptica*, and *Streptococcus equisimilis* were isolated once from different PFTS-SICK pigs (Table 4.2). There were no group differences in the frequency of the above bacteria (Table 4.2).

4.3.3. Detection of viruses

The viral detection results are summarized in Table 4.2. No PCV-2 antigen was detected in lymphoid tissue, stomach, or small and large intestine of any PFTS-SICK or PFTS-HLTHY pig by IHC. All PFTS-SICK and PFTS-HLTHY pigs tested negative for PRRSV, FLUAV, and ACoV-1. A low proportion of PFTS-SICK pigs were positive for RV-A in small intestine by IHC. Enteric calicivirus was detected in at least 1 pig in each group by PCR, and SuHV-2 was detected in all pigs except in 1 PFTS-SICK pig. *Betacoronavirus 1* was present in the tonsils of one-third of the PFTS-SICK pigs, but not in any PFTS-HLTHY and CTRL pig. However, the lungs, kidneys, and small and large intestines were negative for BCoV-1 DNA. Further, when the brain stems (medulla oblongata) of the BCoV-1 tonsil-positive PFTS-SICK pigs were tested for BCoV-1 DNA by PCR, all were negative. All of the pigs were negative for TTV-1, and small numbers of pigs in each group were positive for TTV-2. Despite the identification of these viral agents, there were no statistical group differences in the detection frequencies among PFTS-SICK, PFTS-HLTHY, and CTRL pigs (Table 4.2).

4.3.4. Detection of parasites

Nonsporulated coccidia oocysts were detected in all 3 groups (Table 4.2). No other parasitic oocysts were observed by fecal flotation. No statistical differences in the detection frequencies of coccidia were identified among PFTS-SICK, PFTS-HLTHY, and CTRL pigs (Table 4.2).

4.4. Discussion

The current study aimed to describe the pathological changes in pigs affected by PFTS, and to identify common swine pathogens associated with, and possibly the cause of, PFTS. The most

prevalent lesions in PFTS-SICK pigs were superficial lymphocytic fundic gastritis, atrophic enteritis, superficial colitis, lymphocytic and neutrophilic rhinitis, mild nonsuppurative meningoencephalitis, and thymic atrophy. The GI lesions, especially those present in the stomach, of the PFTS-SICK pigs may explain the clinical presentation of anorexia and wasting. Gastritis, enteritis, and colitis together can cause abdominal discomfort and loss of appetite, and thus feed refusal. However, it is also possible that the GI lesions were secondary to anorexia. Fasting in rats can cause small intestinal villous atrophy.⁴⁰ In pigs, Pittman et al. reported that jejunal atrophic enteritis was induced in 6 out of 6 pigs by 4 days of fasting that began on the day of weaning.¹⁵⁴ In that study, lymphocytic gastritis was observed in 3 out of 6 fasted pigs. The same lesion however, was also seen in 3 out of 6 healthy pigs necropsied on the day of weaning (whether these pigs would be affected by PFTS was not known), 2 out of 6 healthy, and 2 out of 6 PFTS-affected pigs all on 4 days postweaning. By contrast, lymphocytic gastritis was not observed in pigs from a PFTS-free farm 4 days postweaning. The data suggest that in the PFTS-affected farm that Pittman studied, lymphocytic gastritis is a background lesion, regardless of treatment group. The same conclusion cannot be drawn in the current study, however, because superficial lymphocytic fundic gastritis was only observed in PFTS-SICK pigs and not in PFTS-HLTHY pigs. It should be noted that in the Pittman study, a detailed histological description of the lymphocytic gastritis is not available, thus comparison of this lesion to the gastric changes observed in the present study is not possible. The pathogenesis of the thymic atrophy observed in PFTS-SICK pigs is unknown in that it may either be a primary change associated with infection by an immunosuppressive pathogen (e.g., PCV2⁶⁵), or secondary to prolonged sickness or anorexia.⁶⁰

Mild to severe suppurative bronchopneumonia was present in approximately 40% of the

PFTS-SICK pigs. Although bronchopneumonia would have likely contributed to the clinical progression of the affected pigs, it cannot fully explain the clinical signs in all PFTS-SICK pigs. It is more likely that the pneumonia developed secondary to debilitation in some sick animals and was caused by opportunistic bacteria. Due to the difficulty in interpreting the biologic relevance of lesions observed in chronically debilitated pigs, it is suggested that diagnostic efforts focus on pigs in the early stages of illness. Thus, future investigations by the current authors will specifically target clinical pigs within 2 weeks of weaning.

A significant portion of PFTS-SICK pigs had very mild nonsuppurative meningoencephalitis, the cause of which was not determined by the current investigation. The significance of this lesion is uncertain. On the one hand, this may be a potential explanation for the chomping behavior, suspected to be centrally neurological in origin, which was observed in this PFTS-affected farm. On the other hand, inflammation in the histological sections examined, including 13 serial sections of brains from each of 3 PFTS-SICK pigs, was very mild, and may not be clinically relevant. Clinical neurological examination and additional histological examination of brain of additional PFTS-SICK pigs is underway and will help determine if the oral behavior and other clinical signs of PFTS are associated with neurological deficits.

It is noteworthy that certain lesions such as atrophic duodenitis, superficial colitis, and rhinitis were highly prevalent in both PFTS-SICK and PFTS-HLTHY pigs, and the frequencies of these lesions were not statistically different. The presence of these lesions in both groups leads to 3 possible interpretations: 1) atrophic duodenitis, superficial colitis, and rhinitis may be background lesions in pigs postweaning in this farm and not associated with PFTS; 2) these lesions may exist before or at weaning and increase the risk of PFTS; or 3) these lesions may exist before or at weaning and subsequently trigger PFTS in some but not all pigs. The data of

the current investigation does not preferentially point to any of these 3 possibilities.

Several pathogens were only detected in PFTS-SICK pigs including RV-A, BCoV-1, *C. perfringens* type A, *B. bronchiseptica*, *Streptococcus* spp., *H. parasuis*, and *P. multocida*. It is the opinion of the authors that the clinical presentation of PFTS is not consistent with infection by these agents. Further, none of the above pathogens were detected more frequently ($P > .05$ for all) in PFTS-SICK compared to PFTS-HLTHY or CTRL pigs (Table 4.2). Thus, their causal role in PFTS is not supported by the results of the current study.

Rotavirus is a well-known cause of diarrhea and atrophic enteritis. Of the 4 rotavirus-positive pigs, 2 had very mild positive staining and were reported^c as “suspicious.” The antibody against rotavirus used in the current investigation is polyclonal. Although the antibody can react with group A rotavirus, whether it reacts with groups B and C rotavirus is not known. Polymerase chain reaction typing of the rotavirus detected in PFTS-SICK pigs may help diagnosticians understand the role of rotavirus in PFTS. Rotavirus is highly prevalent in swineherds, especially in nursery pigs,^{62,100} and the clinical signs of PFTS are not consistent with rotavirus infection (i.e., lack of consistent diarrhea in PFTS-SICK pigs). The involvement of *Rotavirus B* and *C* in PFTS needs further clarification.

Betacoronavirus 1 can cause vomiting and wasting disease (VWD) in suckling pigs, can frequently be found in the brain stem and ganglions of the GI tract of infected pigs, and this distribution of BCoV-1 can explain the typical GI clinical signs.⁶ *Betacoronavirus 1*, however, was not detected in the GI tract of any PFTS-SICK pig, nor in the brain stem (medulla oblongata) of PFTS-SICK pigs that were BCoV-1–positive in tonsil. *Betacoronavirus 1* is found in tonsil of acutely infected pigs.¹³³ In the current study, however, the PFTS-SICK pigs were chronically

affected at the time of necropsy. Thus, the presence of BCoV-1 in tonsil only and the absence of histological changes in the tonsil fail to explain clinical signs in PFTS-SICK pigs. Although the data from the current investigation does not definitely rule out BCoV-1 as the cause of PFTS, evidence for its causal role is insufficient in the authors' opinion.

Attaching and effacing *E. coli* associated with superficial colitis was observed histologically in both PFTS-SICK and PFTS-HLTHY pigs, and PCR was employed to confirm the presence of AEEC DNA in affected colon. Surprisingly, AEEC DNA was detected in some pigs in all 3 groups, and group differences were not statistically significant. It has been reported that AEEC only causes mild disease in pigs and is usually associated with infection by other pathogens.^{67,86} It is possible that AEEC-associated colitis is a background lesion on this farm with no clinical significance. Alternatively, the underlying colitis associated with AEEC and/or other unidentified etiology could predispose pigs to PFTS.

Detection of PECV in all groups of pigs is consistent with the current understanding of the epidemiology and pathogenesis of PECV infection in swine.²⁰² Porcine enteric calicivirus only causes diarrhea and small intestinal villous atrophy in gnotobiotic piglets following experimental infections⁶¹ and is not associated with diarrhea in naturally exposed piglets.⁶² Accordingly, the role of PECV in PFTS is not determined but unlikely in the authors' opinion.

Although coccidia oocysts (eggs) were identified in all groups, it was not possible to identify the genus and species by morphology. *Cystoisospora suis* and several *Eimeria* spp. can infect pigs, but only *C. suis* can cause prominent disease. *C. suis* typically causes diarrhea and low mortality in 1–3-week-old pigs, which does not match the clinical presentation of PFTS.¹⁰⁶ Older pigs are more resistant to *C. suis*,²⁰⁸ and the PFTS-SICK pigs were at the edge of the susceptible age.

However, the histological presence of coccidia in the PFTS-SICK pigs was clearly associated with enteritis in some cases. Thus, further investigation is needed to not only determine the species, but to determine whether the PFTS-SICK pigs were infected with a highly pathogenic strain or dose of coccidia. Moreover, it is necessary to determine whether *C. suis*/coccidia is a primary or secondary (i.e., sick pigs are more susceptible to the infection) pathogen.

Suid herpesvirus 2 is highly prevalent in suckling and nursery pigs and is not always associated with detrimental clinical signs.⁶³ The present study further confirmed the high prevalence of SuHV-2, as nearly all the pigs, regardless of group, were infected with SuHV-2. Sneezing is the usual manifestation of SuHV-2 infection, and mortality is usually low.¹⁰⁶ Rarely, systemic infection by the virus can cause death.¹⁴⁴ However, SuHV-2 inclusion bodies were not observed in organs outside the nasal cavity in any PFTS-SICK pigs. Thus, the role of SuHV-2 in the pathogenesis of PFTS is not clear but unlikely in the authors' opinion.

In conclusion, the most frequently observed lesions in PFTS-affected pigs were superficial lymphocytic fundic gastritis, atrophic enteritis, superficial colitis, lymphocytic and neutrophilic rhinitis, nonsuppurative meningoencephalitis, and thymic atrophy. Although several pathogens were identified in PFTS-affected pigs, the current diagnostic investigation was not successful in identifying the definite cause of PFTS. There is a lack of compelling evidence to support the causal roles of the following pathogens in PFTS: PRRSV, PCV-2, FLUAV, ACoV-1, RV-A, SuHV-2, PECV, BCoV-1, *C. perfringens*, pathogenic *E. coli*, *B. hyodysenteriae*, *B. pilosicoli*, *Bordetella* spp., *Streptococcus* spp., *H. parasuis*, *P. multocida*, and coccidia.

Table 4.1. Tissues examined histologically from porcine periweaning failure-to-thrive syndrome (PFTS)-affected (PFTS-SICK), age-matched healthy (PFTS-HLTHY) pigs from an affected farm, and healthy pigs selected from 2 unaffected farms (CTRL).

	PFTS-SICK	PFTS-HLTHY	CTRL
Tissues	(n = 18)	(n = 7)	(n = 8)
Respiratory system			
Nasal turbinate	16	6	4
Lung (cranial and caudal)	18	7	8
Bronchus	6	4	0
Cardiovascular system			
Heart	18	7	8
Digestive system			
Tongue	5	3	0
Esophagus	6	4	0
Stomach (fundus)	16	6	8
Duodenum	11	7	8
Jejunum	17	7	8
Ileum	17	7	8
Colon	18	7	8
Pancreas	11	7	8
Liver	18	6	8
Immune system			

	PFTS-SICK	PFTS-HLTHY	CTRL
Tissues	(n = 18)	(n = 7)	(n = 8)
Inguinal lymph node	18	7	8
Mesenteric lymph node	18	7	8
Bronchial lymph node	18	7	8
Spleen	18	7	8
Thymus	17	7	8
Urinary system			
Kidney	18	7	8
Nervous system			
Cerebrum	13	4	8
Cerebellum	13	4	8
Brain stem	9	4	8
Trigeminal ganglion	6	4	0
Glossopharyngeal nerve	6	4	0
Facial nerve	6	4	0

Table 4.2. Frequency (prevalence) of positive diagnostic tests performed to identify common swine pathogens potentially associated with porcine periweaning failure-to-thrive syndrome (PFTS) from PFTS-affected (PFTS-SICK), age-matched healthy (PFTS-HLTHY) pigs from an affected farm, and healthy pigs selected from 2 unaffected farms (CTRL).

Pathogen	Sample	Test	No. positive/No. tested (positive %)			P
			PFTS-SICK K (n = 18)	PFTS-HLTH Y (n = 7)	CTRL (n = 8)	
Bacteria						
<i>Clostridium perfringens</i>	Colon and/or ileum	Culture	4/18 (22.2)	2/7 (28.6)	0/8 (0)	NS
Pathogenic <i>Escherichia coli</i> †	Colon and/or ileum	PCR	8/12‡ (66.7)	2/3 (66.7)	4/7 (50)	NS
Attaching and effacing <i>E. coli</i>	Colon	Histology	8/18 (44.4)	3/7 (42.9)	0/8 (0)	0.06
	Colon	PCR	2/12‡ (16.7)	2/3 (66.7)	2/8 (25)	NS
<i>Pasteurella multocida</i>	Lung	Culture	1/18 (5.6)	0/7 (0)	0/8 (0)	NS
<i>Streptococcus suis</i>	Lung	Culture	1/18 (5.6)	0/7 (0)	0/8 (0)	NS
<i>Haemophilus parasuis</i>	Lung	Culture	1/18 (5.6)	0/7 (0)	0/8 (0)	NS
<i>Bordetella bronchiseptica</i>	Lung	Culture	1/18 (5.6)	0/7 (0)	0/8 (0)	NS
<i>Brachyspira hyodysenteriae</i>	Colon	PCR	0/12‡ (0)	0/3‡ (0)	NT	NS
<i>Brachyspira pilosicoli</i>	Colon	PCR	0/12‡ (0)	0/3‡ (0)	NT	NS
Viruses						
<i>Porcine circovirus-2</i>	Lymphoid, GI	IHC	0/18 (0)	0/7 (0)	NT	NS

Pathogen	Sample	Test	No. positive/No. tested (positive %)			P
			PFTS-SIC K (n = 18)	PFTS-HLTH Y (n = 7)	CTRL (n = 8)	
<i>Porcine reproductive and respiratory syndrome virus</i>	Lung	PCR	0/18 (0)	0/7 (0)	NT	NS
<i>Influenza A virus</i> (H3N2 and H1N1)	Lung	PCR	0/18 (0)	0/7 (0)	NT	NS
<i>Alphacoronavirus 1</i> (<i>Transmissible gastroenteritis virus</i>)	GI	IHC	0/18 (0)	0/7 (0)	NT	NS
<i>Rotavirus A</i>	GI	IHC	4/18 (22.2)	0/7 (0)	NT	NS
Porcine enteric calicivirus	Jejunum, colon	PCR	4/18 (22.2)	1/7 (14.3)	1/8 (12.5)	NS
<i>Suid herpesvirus 2</i> (<i>Porcine cytomegalovirus</i>)	Nasal turbinate	Histology	5/16§ (31.3)	1/6§ (16.7)	0/4§ (0)	NS
	Tonsil, jejunum, colon, kidney, lung	PCR	17/18 (94.4)	7/7 (100)	8/8 (100)	NS
<i>Betacoronavirus 1</i> (<i>Haemagglutinating encephalomyelitis virus</i>)	Tonsil	PCR	6/18 (33.3)	0/7 (0)	0/8 (0)	0.07
	Jejunum, colon, kidney, lung	PCR	0/18 (0)	0/7 (0)	0/8 (0)	NS
	Brain (medulla oblongata)	PCR	0/6! (0)	NT	NT	NA
<i>Torque teno virus 1</i>	Spleen/bone marrow	PCR	0/18 (0)	0/7 (0)	0/8 (0)	NS

Pathogen	Sample	Test	No. positive/No. tested (positive %)			P
			PFTS-SIC K (n = 18)	PFTS-HLTH Y (n = 7)	CTRL (n = 8)	
<i>Torque teno virus 2</i>	Spleen/bone marrow	PCR	3/18 (16.7)	2/7 (28.6)	1/8 (12.5)	NS
Parasites						
Coccidia	Feces	Flotation	3/5 (60)	1/3 (33.3)	3/5# (60)	NS
	Jejunum and/or ileum	Histology	6/18 (33.3)	0/7 (0)	1/8 (12.5)	0.17

* GI = gastrointestinal tract (including stomach, small and large intestines); PCR = polymerase chain reaction; IHC = immunohistochemistry; NT = not tested; NS = not significant ($P > 0.2$); NA = not applicable.

† Pathogenic *E. coli* in the current study is defined as isolates that are positive for 1 or more of the virulence factor genes, namely, AEE, AIDA, Sta, STb, LT, and SLT.

‡ The rest of the pigs in this group were not tested. The same applies to the cells with the same superscript.

§ Nasal turbinate was not sampled in the rest of the pigs in this group. The same applies to the cells with the same superscript.

| These 6 medulla oblongata are from the pigs that tested positive for *Betacoronavirus 1* in tonsils.

The 5 fecal samples were collected from age-matched pigs of 1 CTRL farm, and these pigs were not the same as those for histological evaluation.

Table 4.3. Summary of polymerase chain reaction (PCR) primers and conditions used in the current study.*

Virus	Primers (5'–3')	PCR cycles	PCR reagent	References
<i>Suid herpesvirus 2</i> (<i>Porcine cytomegalovirus</i>)	CMV-1: CCCTGATCTTAAATGACGAGGACGTGAC CMV-2: ACCGTCTGAGAGACTGAACTTCTCTGAC AC	95°C 5 min; 15 cycles of 95°C 1 min, 60°C 1 min, –0.5°C/cycle, 70°C 1 min; 15 cycles of 94°C 45 sec, 52°C 40 sec, –0.5°C/cycle, 70°C 1 min; 10 cycles of 92°C 45 sec, 50°C 30 sec, 70°C 1 min; 70°C 10 min	Qiagen HotStarTaq <i>Plus</i> DNA polymerase (catalog no. 203603)	63

Virus	Primers (5'-3')	PCR cycles	PCR reagent	References
<i>Betacoronavirus 1(Transmissible gastroenteritis virus)</i>	Primary forward: GTTACAGCAAAGGTTAGTCCTGG Primary reverse: AATCTGGTGCCACTGAAGATT Nested forward: TGGATGTTCACTGGTAGTAGC Nested reverse: GGTTGGGTGTCGATGTGTTTCAGC	Primary RT-PCR: 50°C 30 min; 95°C 15 min; 40 cycles of 95°C 30 sec, 63°C 30 sec, 72°C 45 sec; 72°C 10 min Nested PCR: 95°C 5 min; 40 cycles of 95°C 30 sec, 59.7°C 30 sec, 72°C 30 sec; 72°C 10 min	Primary: Qiagen OneStep RT PCR kit (catalog no. 210212) Nested: Qiagen HotStarTaq <i>Plus</i> DNA polymerase (catalog no. 203603)	¹⁷⁹

Virus	Primers (5'-3')	PCR cycles	PCR reagent	References
Porcine enteric calicivirus	Forward 1: GATTACTCCAAGTGGGACTCCAC Forward 2: GATTACTCCAGGTGGGACTCCAC Forward 3: GATTACTCCAGGTGGGACTCAAC Forward 4: GATTACTCCACCTGGGATTCAAC Forward 5: GATTACTCCACCTGGGATTCCAC Reverse 1: TGACAATGTAATCATCACCATA Reverse 2: TGACGATTCATCATCACCATA Reverse 3: TGACGATTCATCATCCCCGTA	50°C 30 min; 95°C 15 min; 40 cycles of 95°C 30 sec, 59°C 30 sec, 72°C 30 sec; 72°C 10 min	Qiagen OneStep RT PCR kit (catalog no. 210212)	²⁰³

Virus	Primers (5'–3')	PCR cycles	PCR reagent	References
<i>Porcine reproductive and respiratory syndrome virus</i>	Refer to user manual	Refer to user manual	Tetracore NextGen NA/EU PRRSV multiplex RT-PCR reagents (catalog no. TC-9039-080)	Refer to user manual
<i>Influenza A virus</i> (H1N1 and H3N2)	Forward: AGATGAGTCYTCTAACCGAGGTCG Reverse: TGCAAARACAYYTTCMAGTCTCTG Probe: FAM-TCAGGCCCCCTCAAAGCCGA-TAM RA	45°C 10 min, 95°C 10 min; 45 cycles of 94°C 1 sec, 60°C 30 sec	Qiagen OneStep RT PCR kit (catalog no. 210212)	¹⁸⁴

Virus	Primers (5'-3')	PCR cycles	PCR reagent	References
<i>Torque teno virus 1</i>	Primary forward: TACTACTCCGGGTTTCAGGAGGCT Primary reverse: ACTCAGCCATTCGGAACCTCAC Nested forward: CAATTTGGCTCGCTTCGCTCGC Nested reverse: TACTTATATTCGCTTTCGTGGGAAC	For both primary and nested: 95°C 5 min; 40 cycles of 94°C 30 sec, 52°C 30 sec, 72°C 30 sec; 72°C 10 min	Fermentas Taq polymerase (catalog no. EP0406)	⁹²
<i>Torque teno virus 2</i>	Primary forward: AGTTACACATAACCACCAAACC Primary reverse: ATTACCGCCTGCCCGATAGGC Nested forward: CCAAACCACAGGAAACTGTGC Nested reverse: CTTGACTCCGCTCTCAGGAG	For both primary and nested: 95°C 5 min; 40 cycles of 94°C 30 sec, 52°C 30 sec, 72°C 30 sec; 72°C 10 min	Fermentas Taq polymerase (catalog no. EP0406)	⁹²

Virus	Primers (5'–3')	PCR cycles	PCR reagent	References
Virulence factors of <i>Escherichia coli</i> (Eae, AIDA1, STa, STb, LT, and SLT)	Eae –Forward: ATCTTCTGCGTACTGCGTTCA Eae – Reverse: CATTATGGAACGGCAGAGGT AIDA 1—Forward: ACAGTATCATATGGAGCCA AIDA 1—Reverse: TGTGCGCCAGAACTATTA STa—Forward: TCCCCTCTTTTAGTCAGTCAACTG STa—Reverse: GCACAGGCAGGATTACAACAAAGT STb—Forward: GCAATAAGGTTGAGGTGAT STb—Reverse: GCCTGCAGTGAGAAATGGAC LT—Forward: TTACGGCGTTACTATCCTCTCTA LT—Reverse: GGTCTCGGTCAGATATGTGATTC	94°C 3 min; 35 cycles of 94°C 30 sec, 60°C 30 sec, 72°C 30 sec; 72°C 10 min	Fermentas Taq polymerase (catalog no. EP0406)	PDS Inc. (Canada)

Virus	Primers (5'–3')	PCR cycles	PCR reagent	References
<i>Brachyspira hyodysenteriae</i> and <i>Brachyspira pilosicoli</i>	<p><i>B. hyodysenteriae</i>—Forward: TATAAAGGGCGGGCAAATGT</p> <p><i>B. hyodysenteriae</i>—Reverse: TGGAGGAGTGGTAGCTGATAAAA</p> <p><i>B. pilosicoli</i>—Forward: AGAGGAAAGTTTTTTCGCTTC</p> <p><i>B. pilosicoli</i>—Reverse: TCCGCCTACTCACCTTTAC</p>	94°C 3 min; 35 cycles of 94°C 30 sec, 62°C 30 sec, 72°C 30 sec; 72°C 10 min	Fermentas Taq polymerase (catalog no. EP0406)	<i>B. hyodysenteriae</i> : 104; <i>B. hyodysenteriae</i> : 132

Table 4.4. Summary of the primary antibodies used in the current study

Pathogens	Primary antibodies (dilution)	Source
<i>Porcine circovirus-2</i>	Rabbit anti-PCV2 (1:1,000)	Dr. G. Allan, Belfast, Northern Ireland
<i>Rotavirus A</i>	Rabbit anti-porcine rotavirus (1:1,000)	Vaccine and Infectious Disease Organization (VIDO), Saskatoon, SK, Canada
<i>Alphacoronavirus 1(Transmissible gastroenteritis virus)</i>	Pool of monoclonal antibodies 25H7, 14G9, 14F10 (1:1,000)	Dr. Linda Saif, Ohio State University, Columbus, OH

Table 4.5. Frequency of lesions found in porcine periweaning failure-to-thrive syndrome (PFTS)-affected (PFTS-SICK), age-matched healthy (PFTS-HLTHY) pigs from an affected farm, and healthy pigs selected from 2 unaffected farms (CTRL).*

Lesions	PFTS-SICK	PFTS-HLTHY	CTRL	<i>P</i> value‡
	(<i>n</i> = 18)	(<i>n</i> = 7)	(<i>n</i> = 8)	
Rhinitis	14/16† (87.5) ^a	6/6† (100) ^a	1/4† (25) ^b	0.006
Bronchopneumonia	7/18 (38.9) ^a	0/7 (0) ^b	0/8 (0) ^b	0.03
Gastritis	16/16† (100) ^a	0/6† (0) ^b	0/8 (0) ^b	<0.0001
Duodenitis	11/11† (100) ^a	6/7 (85.7) ^a	0/8 (0) ^b	<0.0001
Jejunitis	17/17† (100) ^a	1/7 (14.3) ^b	0/8 (0) ^b	<0.0001
Ileitis	16/17† (94.1) ^a	0/7 (0) ^b	0/8 (0) ^b	<0.0001
Colitis	18/18 (100) ^a	5/7 (71.4) ^a	1/8 (12.5) ^b	<0.0001
Thymic atrophy	15/17† (88.2) ^a	0/7 (0) ^b	0/8 (0) ^b	<0.0001
Nephritis	4/18 (22.2)	0/7 (0)	0/8 (0)	0.22
Fatty liver	5/17† (29.4)	1/6† (16.7)	0/8 (0)	0.23
Meningoencephalitis	6/13† (46) ^a	0/4† (0) ^b	0/8 (0) ^b	0.03

* Numbers in parentheses are percentages.

† When denominators are less than *n*, the rest of the samples were not available.

‡ Frequency values with different superscripts within a row are statistically different (*P* < 0.05).

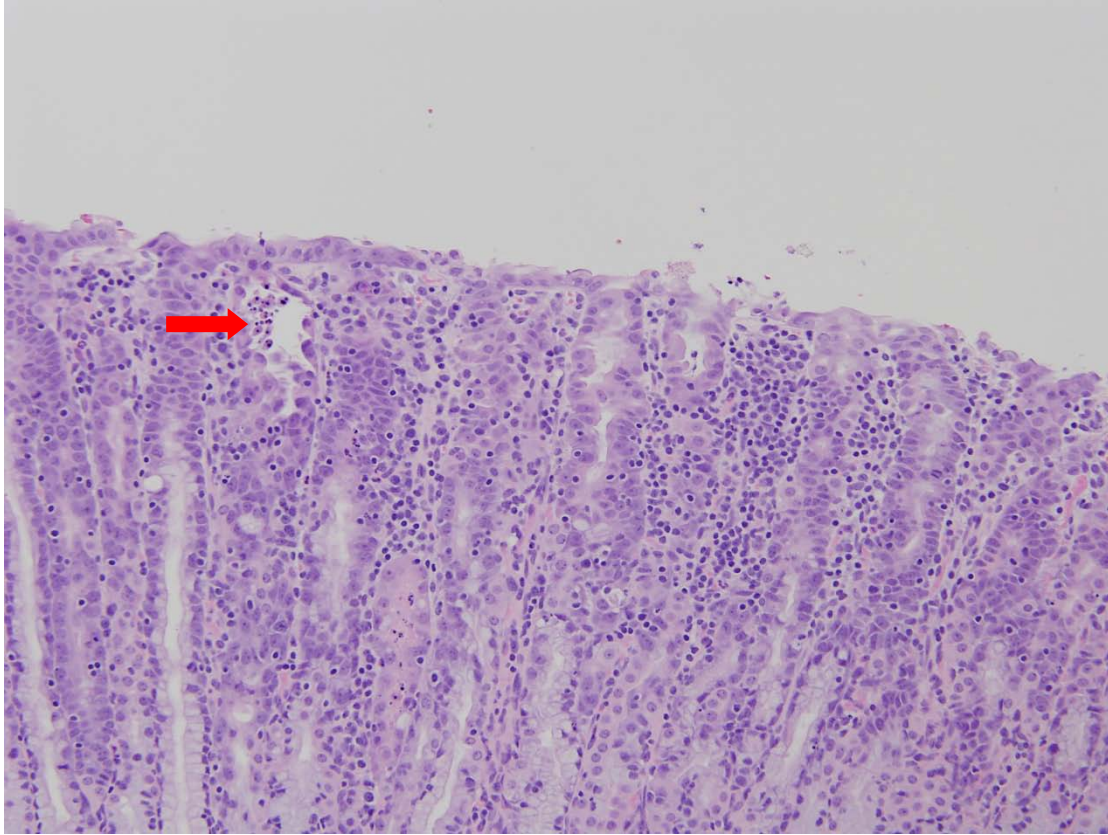


Figure 4.1A. Fundus from a PFTS-SICK pig. The superficial epithelial cells are attenuated with a loss of cytoplasmic secretory material. Moderate numbers of lymphocytes and plasma cells are infiltrating in the lamina propria. Occasionally, some glandular cells are necrotic (arrow) with the surviving cells in the same acinus regenerating.

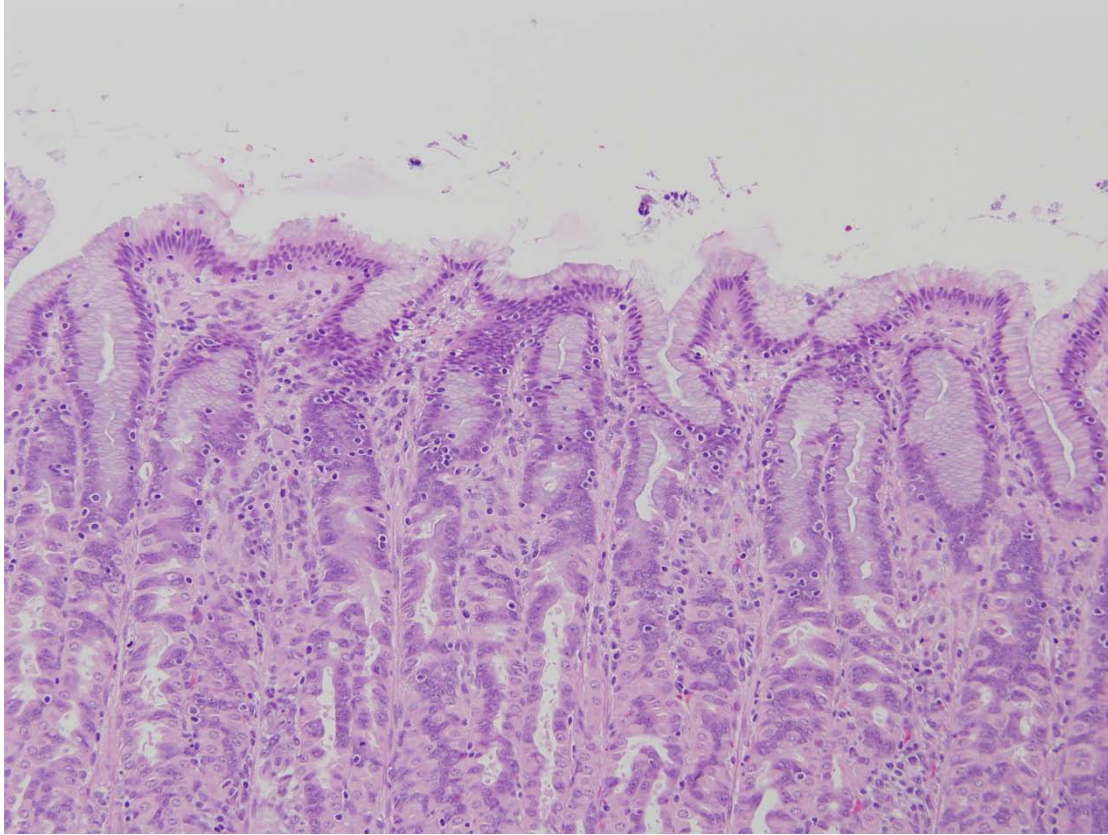


Figure 4.1B. Fundus from a PFTS-HLTHY pig showing abundant cytoplasmic secretory material in the superficial epithelium and limited numbers of immune cells in the lamina propria.

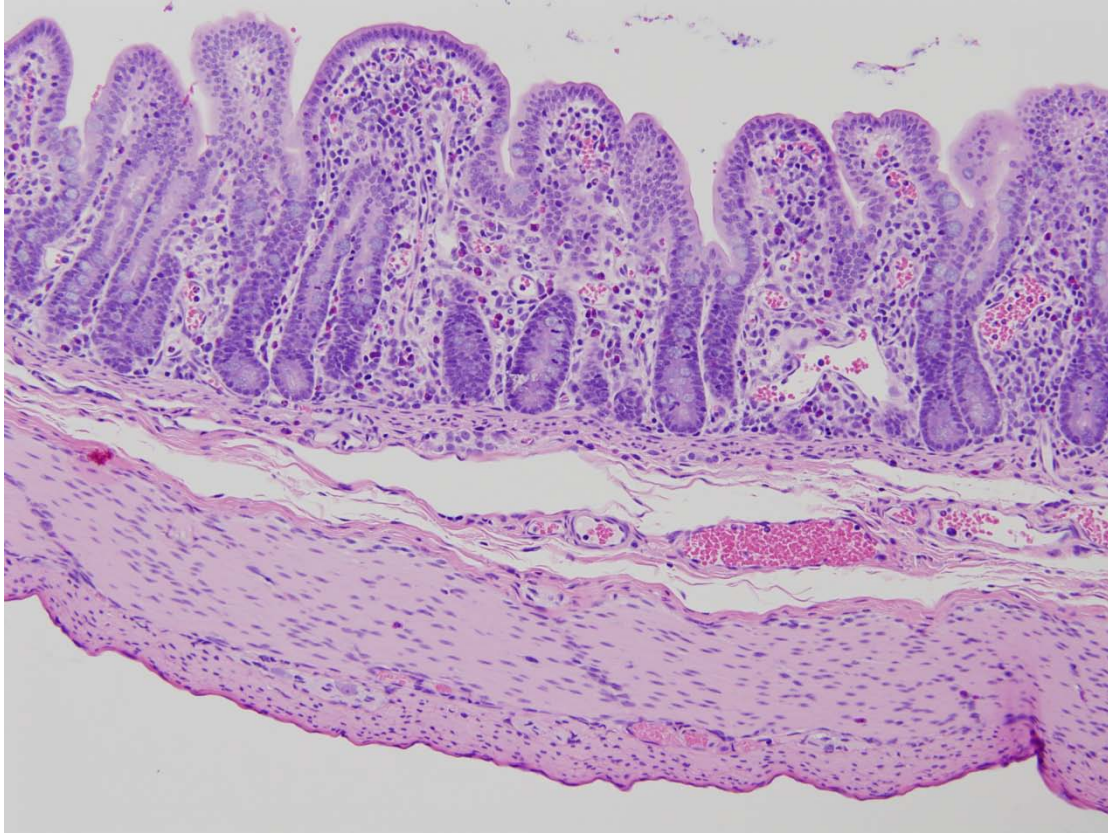


Figure 4.2A. Ileum from a PFTS-SICK pig. The villi are severely shortened, with a villi-to-crypt ratio of less than 1:1. The total thickness of the intestinal wall is also decreased.

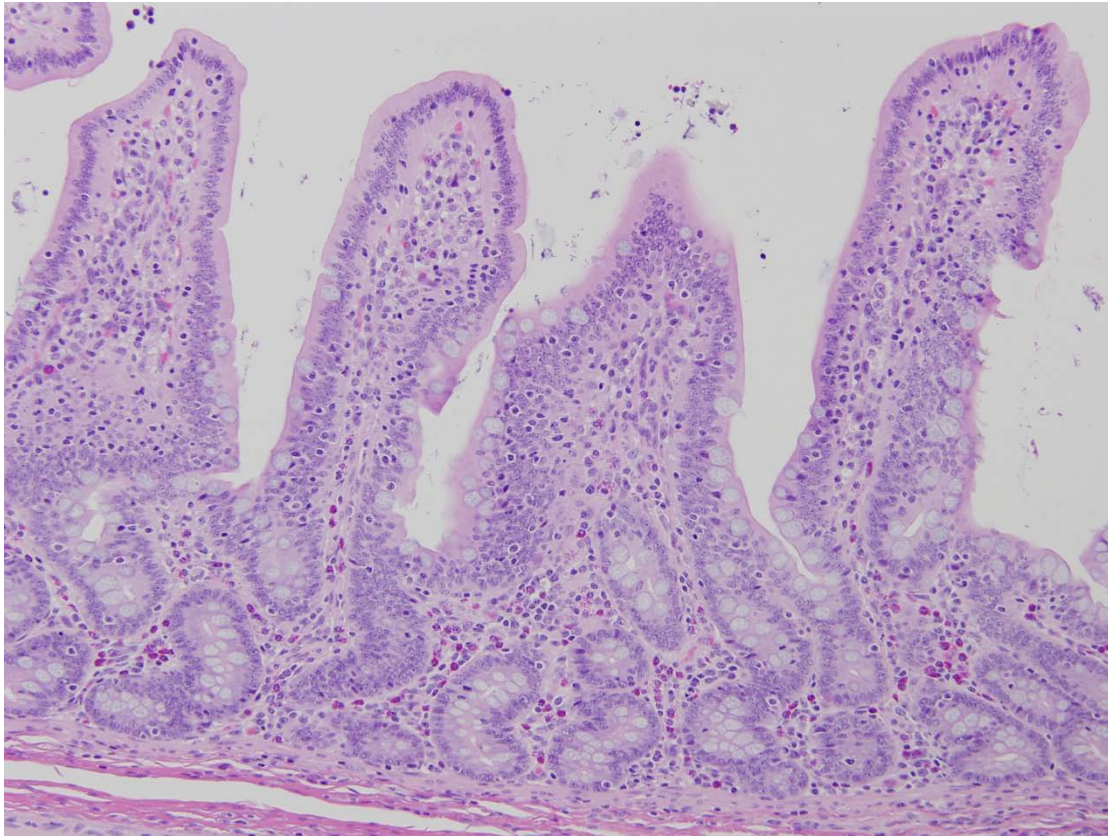


Figure 4.2B. Ileum from a PFTS-HLTHY pig, taken in the same magnification as A, shows the normal villi length of a nursery pig.

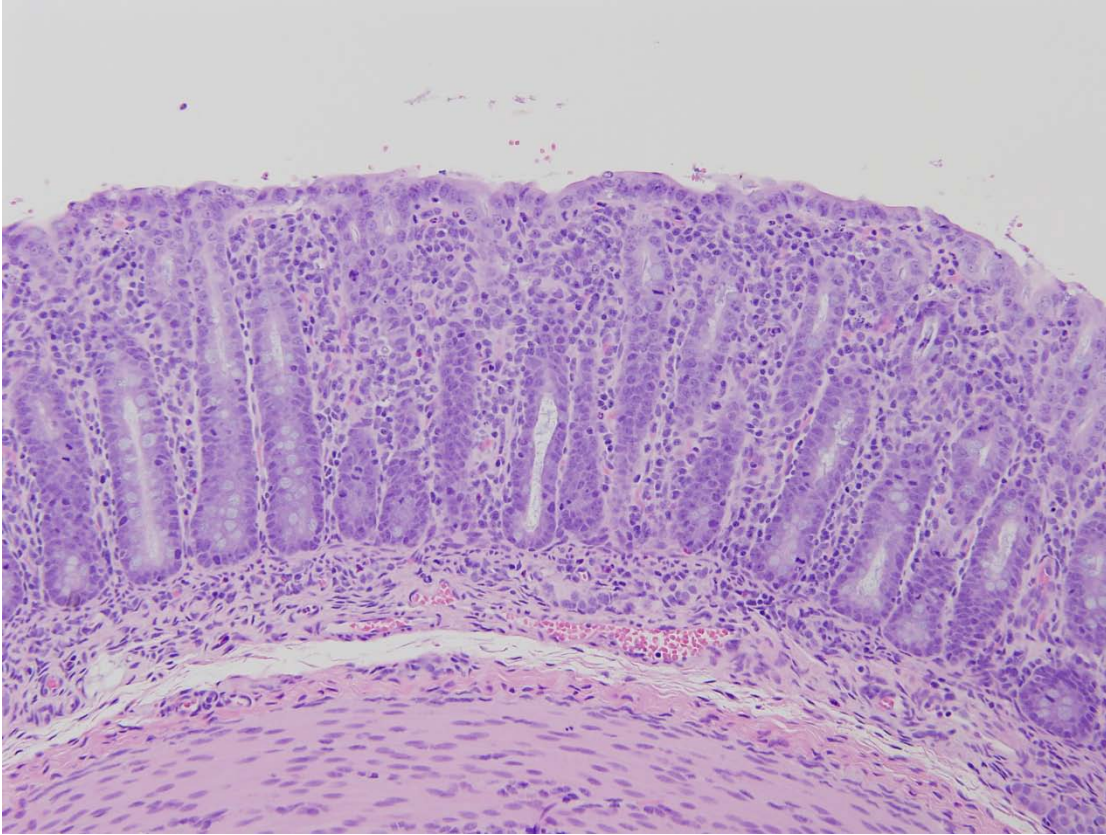


Figure 4.3A. Colon from a PFTS-SICK pig. There is a decreased number of goblet cells. Multifocally, the superficial epithelial cells are attenuated with a loss of cellular polarity. Moderate numbers of lymphocytes, plasma cells, and macrophages are infiltrating in the lamina propria.

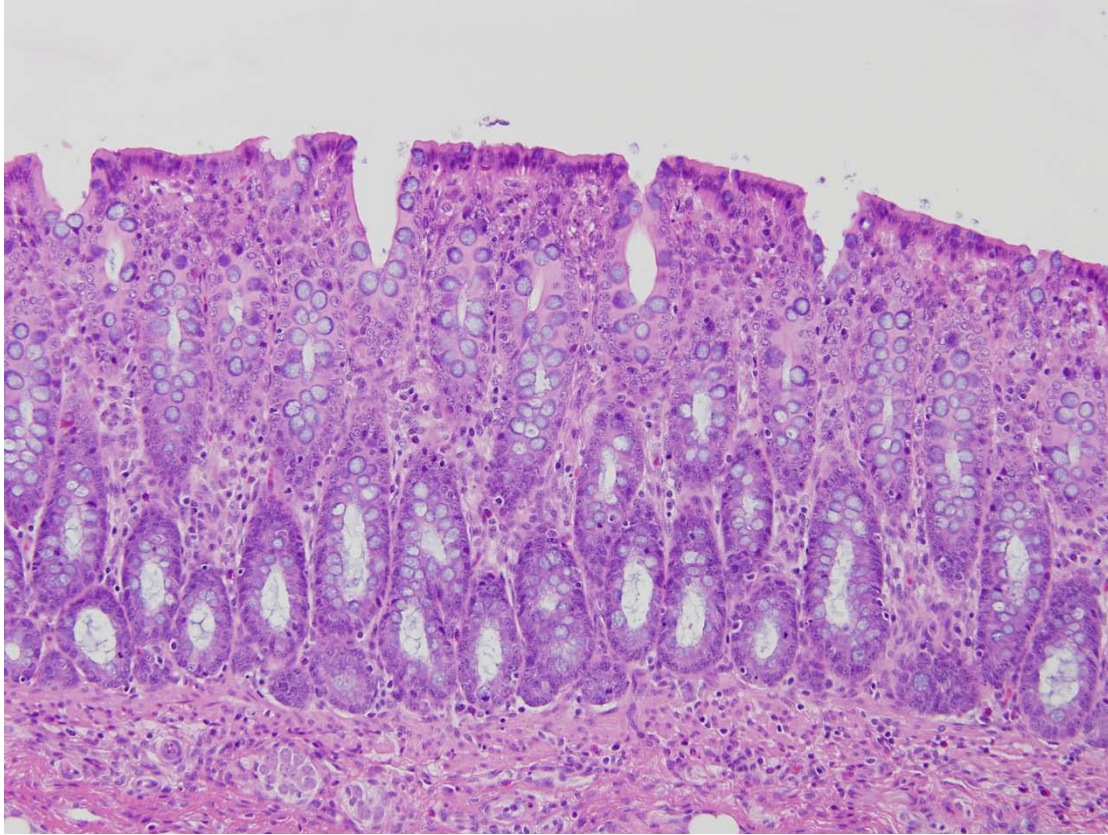


Figure 4.3B. Colon from a CTRL pig showing normal numbers of goblet cells and polarity of the superficial epithelial cells.

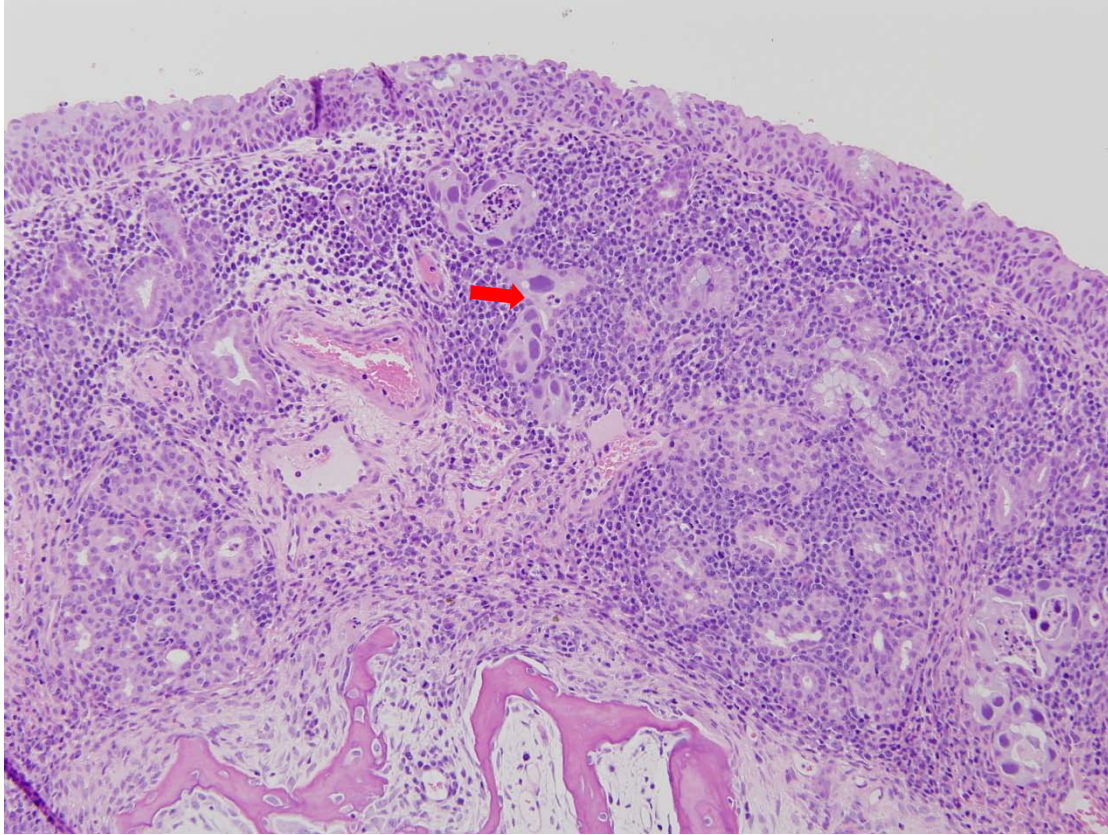


Figure 4.4. Nasal mucosa from a PFTS-SICK pig. Large numbers of inflammatory cells are infiltrating the lamina propria. Occasionally, the nasal glandular cells contain large, basophilic, intranuclear inclusion bodies (arrow), consistent with porcine cytomegalovirus.

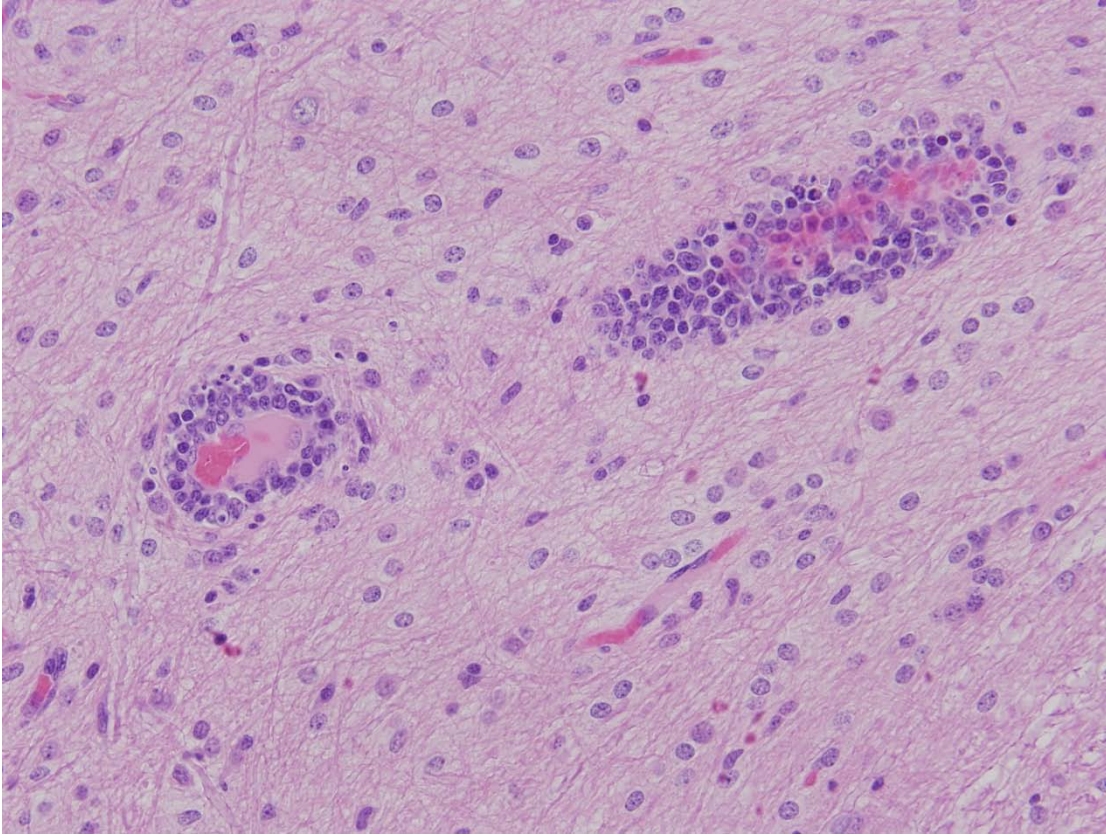


Figure 4.5. Cerebellum of a PFTS-SICK pig. Two lymphocytic perivascular cuffing are shown in the white matter of the cerebellum.

5. Pathological features and proposed diagnostic criteria of porcine periweaning failure-to-thrive syndrome (PFTS).

The investigation from Chapter 4 revealed several highly prevalent lesions, but whether these are consistent between different farms affected by PFTS is unknown. This was considered a very important question. In Chapter 5, a second investigation involving 8 farms is presented. The results confirmed that the pathological findings among different farms are consistent. This finding further justifies the proposal of PFTS as a clinical syndrome. Consistent with the results of the previous study (Chapter 4), there is no evidence that any tested pathogen in Chapter 5 is causally associated with PFTS.

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Huang, Y. and J.C.S. Harding, Pathological features and proposed diagnostic criteria of porcine periweaning failure-to-thrive syndrome (PFTS). *Vet Pathol*, 2013. Submitted, under revision.

Huang and Harding performed farm visits, necropsies, data analysis and manuscript writing.

Huang evaluated the histopathology and performed all laboratory tests.

5.1. Introduction

Porcine periweaning failure-to-thrive syndrome (PFTS) is a clinical syndrome of anorexia and progressive debilitation affecting nursery pigs within 2-3 weeks of weaning.⁷⁹ Although sporadic “post-weaning starve-outs” have existed in the swine industry for many years, it was not until 2008 that outbreaks of “starve-outs” accompanied by substantial increases in nursery mortality were first recognized as a distinct clinical syndrome.^{42,57} Early reports of the syndrome used various terms such as “postweaning catabolic syndrome”, “postweaning wasting-catabolic syndrome”, “failure to thrive syndrome” and “postweaning fading pig-anorexia syndrome”^{42,64,79} until the consensus name “PFTS” was adopted in 2010.⁷⁹ Many nutritional, environmental and medical interventions have been attempted to reduce the impact of PFTS, but none have proven effective.^{42,57,79} A recent survey conducted in North America estimated the flow-prevalence of PFTS to be about 4% and the four most commonly reported clinical signs were anorexia, loss of body condition, prolonged standing and repetitive oral behavior.¹³⁸ The current status of PFTS on other continents is unknown, but a recent report suggests it may be present in Spain.¹⁷⁸

At present, PFTS diagnosis is based on fulfillment of the proposed case definition and exclusion of other common swine diseases. As the etiology is not known, the case definition is mainly based on clinical presentation paraphrased as follows: *PFTS is characterized clinically by the progressive debilitation of newly weaned pigs within 2 to 3 weeks of weaning, resulting in variable morbidity but high case fatality, with repetitive oral behaviors such as chomping and chewing observed in affected pigs, without residual illness from the suckling phase or discernible and detrimental infectious, nutritional, managerial and environmental factors that can explain the clinical symptoms.*⁷⁹

A number of diagnostic investigations in clinically affected farms have been completed to date by our group⁷⁶ and others.^{154,178} These investigations have identified a number of gross and histopathological lesions that may be associated with PFTS, including thymic atrophy, superficial gastritis, small intestinal villous atrophy, superficial colitis, non-suppurative meningoencephalitis, neutrophilic lymphoplasmacytic rhinitis and bronchopneumonia. In addition, the involvement of common swine pathogens including porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus-2 (PCV2), influenza A virus (FLUAV), transmissible gastroenteritis virus, (TGEV), *Mycoplasma hyopneumoniae* (Mhyo), *Brachyspira hyodysenteriae*, and *Brachyspira pilosicoli* was ruled out. However, a number of swine pathogens, such as porcine cytomegalovirus (PCMV), porcine enteric calicivirus (PECV), swine Torque Teno virus 2 (TTV2), pathogenic *Escherichia (E.) coli*, rotavirus (RV-A), haemagglutinating encephalomyelitis virus (HEV) and coccidian, were detected in affected pigs, age-matched unaffected pigs, or both depending on the investigation.^{76,154} Given that the investigations completed to date were limited to a few farms, were conducted by different investigators, and had insufficient experimental power and scope to be conclusive, the objectives of this study were to characterize the pathology and potential pathogens associated with PFTS more conclusively using a larger scale case-control investigation involving North American farms.

5.2. Methods

5.2.1. Farm visits and sample collection

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Between

July 2011 and April 2013, the authors were contacted by a number of swine veterinarians in Canada and the USA who reported client farms with clinical signs typical of PFTS. Relevant aspects of the history, clinical signs and health status were obtained, and farms appearing to fulfill the proposed clinical definition of PFTS⁷⁹ were investigated further. Recent diagnostic reports, when available, were reviewed and farms that had undertaken no recent diagnostics were asked to submit 2-3 characteristic pigs for necropsy examination at their local diagnostic laboratories to verify that no underlying known diseases were responsible for the clinical presentation (elevated morbidity/mortality, anorexia, weight loss within 2-3 weeks of weaning). Eight farms were included in the study (Table 5.1), all having PCV2 vaccination programs in place. The authors performed farm visits and made clinical observations.

During the visits to the 8 selected farms, it was determined that 5 farms (farm 1-5) completely fulfilled the proposed PFTS clinical definition.⁷⁹ These were designated as “PFTS” farms. However, the CASE pigs in two other farms (farm 6 and 7) did not demonstrate progressive debilitation (emaciation and wasting) during the farm visits, despite the fact that increased nursery mortality was reported by the herd veterinarian prior to the visit. CASE pigs from these two farms were selected on the basis of demonstrating a flat or hollow abdomen, lack of interest in the available feed and repetitive oral behavior (chomping). In farm 8, necropsy examination revealed bronchopneumonia of various severities in all CASE but no CTRL pigs. As these latter three farms did not meet the proposed PFTS case definition fully, specifically the lack of progressive debilitation in CASE pigs of farms 6 and 7, and the presence of bronchopneumonia in all CASE pigs from farm 8, they were designated “partial farms” (partially fulfilling the case definition). As a result, pigs used in this study are further divided into 4 subgroups, 1) affected pigs from PFTS-farms (PFTS-CASE); 2) non-affected pigs from PFTS-farms (PFTS-CTRL); 3)

affected pigs from partial-farm (partial-CASE); and 4) non-affected pigs from partial-farm (partial-CTRL). The partial-farms were included in the analysis because they might represent either less severe PFTS or a useful comparison.

Pigs that met the clinical definition (thin to emaciated, presence of repetitive oral behavior, no other signs of overt disease) most convincingly were identified and assigned as CASE.

Non-affected pigs from the same batch were selected conveniently as controls (CTRL). All pigs were weaned less than 15 days. From each farm, 8 or 9 CASE and 4 CTRL pigs blocked by number of days since weaning were identified, transported to the local diagnostic laboratory and held overnight with feed and water. The following day, animals were humanely euthanized using intravenous barbiturate and a comprehensive necropsy examination was performed.

Formalin-fixed and fresh tissues were collected for routine histological examination and further diagnostic tests. Tissues collected included cerebrum, cerebellum, brain stem, trigeminal ganglion, salivary gland, lung, heart, thoracic thymus, stomach (fundus and pylorus), duodenum, jejunum, ileum, spiral colon, cecum, pancreas, liver, kidney, adrenal gland, second rib, tonsil, bronchial lymph node, mesenteric lymph node, superficial inguinal lymph node and spleen.

Fresh tissues were stored at -80°C before further use. Histological examinations were carried out by the author (YH) while blinded to the identities of the animals. Additionally, the lengths and depths of 10 randomly selected villi and crypts in ileum were measured under 10X microscopic fields by a micrometer.

5.2.2. Nucleic acid extraction and reverse transcription of RNA

DNA and RNA of tonsil, brain, lung, ileum and stomach were extracted using the Qiagen AllPrep DNA/RNA Mini Kit (Toronto, ON, Canada) according to the manufacturer's instruction. Reverse

transcription of the extracted RNA was then performed using the Bio-Rad iScript™ cDNA Synthesis Kit (Mississauga, ON, Canada) according to the manufacturer's instruction. DNA, RNA and cDNA were stored at -80°C before being tested for different pathogens by polymerase chain reactions (PCR).

5.2.3. PCR detection of common and relevant swine pathogens

Detection of nucleic acid of HEV in tonsil,^{76,179} porcine enteroviruses CPE group 1, 2 and 3 (PEV-1, 2 and 3) in brain²¹⁰, PECV in ileum^{76,203}, TGEV in ileum²⁰¹, PCV2 in tonsil⁵⁵, Mhyo in lung¹¹⁴, and *Helicobacter-pylori*-like (Hp) and *Helicobacter-heilmannii*-like (Hh) in stomach²⁸ were performed according to previously published assays using the Qiagen HotStarTaq Plus DNA Polymerase Kit (Toronto, ON, Canada). PCR detection of FLUAV in lung tissue was performed as previously described^{76,184} using the Qiagen QuantiFast Probe PCR Kit according to the manufacturer's instructions (Toronto, ON, Canada). Detection of PRRSV RNA from lung was performed using the Tetracore EZ-PRRSV™ Kit (Rockville, MD, USA) according to manufacturer's instructions. PCR detection of RV-A, B and C were performed by the Veterinary Diagnostic Services Laboratory of Government of Manitoba (Winnipeg, MB, Canada) using a protocol developed by the Veterinary Diagnostic Laboratory of University of Minnesota.¹¹²

5.2.4. Statistical analyses

Generalized Estimating Equations (GEE) adjusted for clustering effects of farm were employed to build logistic regression models, in which the outcomes were the presence or absence of specific lesions and pathogens. The predictors for all models were farm-type (PFTS- or partial-farms), pig-type (CASE or CTRL) and the interaction of these two factors. Post hoc

pairwise comparisons of the estimated means of each combination of farm-type and pig-type were performed when the interaction term was statistically significant, or when both farm-type and pig-type were significant (Table 5.2). When the interaction of the initial GEE was significant (i.e. superficial gastritis, duodenal villous atrophy, jejuna villous atrophy and ileal villous atrophy), or the prevalence of the lesion was obviously higher in PFTS-CASE pigs than in the others (i.e. thymic atrophy and colitis), further analyses were completed comparing the odds of lesions in PFTS-CASE versus all other groups (Table 5.2). When the prevalence of lesions were obviously higher in pigs from PFTS-farms than in those from partial-farms (i.e. encephalitis and rhinitis), further analyses were pursued to compare the odds of lesions between PFTS-farms versus partial-farms (Table 5.2). Linear regressions were built by GEE to predict ileal villous lengths, crypt depths and villi-to-crypt (V/C) ratios, with the predictors being farm-type, pig-type and the interaction term. The raw residues of the linear regression models were checked for normality and homogeneity of the variances (Table 5.3). All statistical analyses were performed using IBM SPSS Statistics version 21. *P* values less than 0.05 were regarded as statistically significant. 95% confidence intervals (CI) of sensitivities and specificities were calculated as described.¹⁹⁴

5.3. Results

The most notable gross lesions in PFTS-case pigs were thymic atrophy and empty gastrointestinal tracts (GIT). Histological lesions that were more than 60% prevalent in PFTS-CASE were: superficial gastritis, small intestinal villous atrophy of duodenum, jejunum and ileum, thymic atrophy and suppurative or lymphoplasmacytic rhinitis (Table 5.2). The gastritis was mainly observed in fundic stomach. Compared to normal (Figure 5.1), the superficial foveolar cells of affected fundus lacked or had reduced cytoplasmic mucus, were

attenuated, low cuboidal, squamous or multifocally absent (erosion) (Figure 5.2). The same epithelial changes occasionally extended deeper, affecting the mucous neck cells and upper glands. Increased numbers of lymphocytes were observed in the lamina propria of some pigs, without formation of lymphoid follicles. However, at times these changes were subtle and difficult to distinguish confidently from normal. Small intestinal villous atrophy was defined in this study by subjective evaluation of the general presence of V/C ratios of less than 1. Whereas normal nursery pigs generally have small intestinal V/C ratios greater than 1 (Figure 5.3), the ratios in PFTS affected intestines were usually less than one (Figure 5.4). It should be emphasized that this was a subjective estimation of the histological image, which means the V/C ratio cannot be strictly applied to each individual villus. The measured villous lengths and crypt depths in ileum demonstrated that PFTS-CASE pigs had shorter villi and lower V/C ratios than all other pigs (Table 5.3). Crypt depths of PFTS-CASE pigs were not increased compared to other groups (Table 5.3). Further, when compared to the V/C ratios of ileum calculated from measured villous lengths and crypt depths, the subjective evaluations and judgments of villous atrophy were highly sensitive and specific (Table 5.4). Epithelial cells on the villous tips were often cuboidal and occasionally squamous. Coccidia of different life cycle stages were inconsistently observed in jejunal or ileal epithelium in 3 PFTS-farms (5/8 CASE and 1/4 CTRL from farm 2, 1/4 CTRL pig from farm 4, 1/8 CASE pig from farm 5) and 1 partial-farm (2/8 CASE from farm 6). Compared to normal thymus (Figure 5.5), thymic cortex of PFTS-CASE pigs demonstrated markedly reduced numbers of lymphocytes (Figure 5.6). As a result, there was moderate to severe reduction in thickness of the thymic cortex in PFTS-CASE pigs but the medulla was relatively unaffected (Figure 5.6).

In the nasal cavities and sinuses of the pigs affected by rhinitis, large numbers of neutrophils

were mixed with mucous without observable bacteria. Moderate to large numbers of lymphocytes and plasma cells infiltrated the submucosa of the nasal turbinate, which was either diffusely or multifocally surrounding submucosal glands. Characteristic intranuclear cytomegaloviral inclusion bodies in the submucosal glandular epithelial cells were an inconsistent finding observed in one PFTS-farm (5/8 CASE from farm 4) and two partial-farms (1/8 CASE and 1/4 CTRL from farm 7 and 1/4 CASE from farm 8). Less frequently observed lesions included superficial colitis, bronchopneumonia, hepatic cellular fatty change and multifocal lymphoplasmacytic nephritis, which ranged in prevalence but were unrelated to pig-type (Table 5.2).

Lesions that were more than 20% prevalent in PFTS-CASE pigs were subjected to statistical analyses. PFTS-CASE pigs had significantly higher odds of showing superficial gastritis (OR=16.7; 95% CI: 6.8-40.6) small intestinal villous atrophy of duodenum (OR=28.7; 95% CI: 7.7-107), jejunum (OR=67.4; 95% CI: 7.0-651), ileum (OR=56.3; 95% CI: 14.1-224), thymic atrophy (OR=30.1; 95% CI: 6.0-152) and superficial colitis (OR=14.6; 95% CI: 2.0-107) than all other pig-types (Table 5.2). It is noteworthy that some thymuses from PFTS-CASE pigs were not available for histological examination. The most likely reason for this was that total thymic atrophy was apparent on gross examination and hence no tissues were available for histopathological evaluation. The pathological status of the 31 PFTS-CASE thymuses included in Table 5.2 represents combined histological and gross examination results. Unfortunately, gross pathological data on 9 thymuses is missing, thus it is impossible to verify their pathological status, although they were most likely severely atrophied and not easily visible at necropsy.

Twenty-nine PFTS-CASE and 43 non-PFTS pigs (PFTS-CTRL, partial-CASE, partial-CTRL) had stomach, small intestine and thymus available for histopathological evaluation. Using the

presence of at least two of superficial gastritis, small intestinal villous atrophy and thymic atrophy as a diagnostic criterion (i.e. having 2 or 3 of these 3 lesions), the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of correctly identifying PFTS-CASE was 100%, 67.4%, 67.4% and 100% (Table 5.5). When using the presence of all three of the above lesions as a diagnostic criterion, sensitivity and NPV decreased moderately to 85.7% and 90.7, respectively, while the specificity and PPV increased moderately to 84.8% and 77.4%, respectively (Table 5.5).

For rhinitis and encephalitis or trigeminal neuritis, the pig-type x farm-type interactions were not significant in the initial GEE, and statistical analysis was used to compare PFTS to partial-farms. All pigs from PFTS-farms had significantly higher odds of having suppurative or lymphoplasmacytic rhinitis and non-suppurative encephalitis or trigeminitis than all pigs from partial-farms. (Table 5.2) The odds of pneumonia, liver and renal lesions did not differ by farm-type or pig-type. (Table 5.2)

RV-A, B and C, HEV, PEV-1, 2 or 3 were detected variably by PCR in both pig-types and farm-types (Table 5.6). The odds of infection of RV-A and B, HEV, PEV-1, 2 or 3 did not differ by farm-type or pig-type ($P>0.05$). RV-C had higher odds of detection in pigs from PFTS-farms compared to those from partial-farms ($P=0.023$; OR=3.3 [95% CI: 1.2-9.3]). One PFTS-CASE (from farm 1) tested positive for PCV2, and all pigs in this study tested negative for PRRSV, FLUAV, PECV, TGEV, Mhyo, Hp and Hh. The detection of HEV and PEVs were not associated with the presence of non-suppurative meningoencephalitis or trigeminal neuritis ($P>0.05$ for all).

5.4. Discussion

The results of the current investigation provide new knowledge on the pathology and

microbiology of PFTS. Since a detailed discussion of the significance of the lesions associated with PFTS was made previously,⁷⁶ our goal in this manuscript is to expand understanding rather than reiterate a similar discussion.

Superficial colitis was highly prevalent in our previously reported investigation in both PFTS and non-affected groups.⁷⁶ This suggested colitis was a background lesion in the farm investigated. In the current study, the overall prevalence of superficial colitis in PFTS-CASE was lower (31%), but the prevalence varied widely by farm (0 – 67%). This further supports the observation that colitis is not a consistent finding of PFTS and does not likely contribute significantly to the pathogenesis.

In our previous diagnostic investigation⁷⁶ we also reported that rhinitis and non-suppurative meningoencephalitis were lesions possibly associated with PFTS. In the present study, rhinitis and non-suppurative meningoencephalitis or trigeminal neuritis were not related to pig-type (CASE or CTRL) but these lesions were at higher odds of being present in pigs from PFTS-farms than partial-farms. Whether they are risk factors for farms developing PFTS is unknown and needs to be verified in a larger study.

It is noteworthy that the high prevalences of superficial gastritis, small intestinal villous atrophy and thymic atrophy of PFTS-CASE pigs are largely consistent with those we have previously reported.⁷⁶ This indicates that the pigs affected by PFTS were not only clinically similar, but also shared similar pathological processes.

Thymic atrophy and small intestinal villous atrophy can be caused both by lack of feed intake,^{60,154} as well as by infection with an organism causing primary atrophy associated with apoptosis or necrosis of thymic lymphocytes or intestinal villi. Unfortunately, there is little

published data describing the effects of anorexia, fasting or starvation on GIT pathology of pigs. It had been described that gastric erosion and ulceration occurred in mice and rats after food deprivation.^{4,123,140} Interestingly, the locations of lesions differed in these two species, in that mice developed these lesions in the secretory portion of the stomach (fundus)¹⁴⁰, but rats tended to have the lesions in the non-secretory parts (forestomach of rats, equivalent to pars esophagea of pigs).^{4,123} In pigs, gastric ulcers associated with feed disruption almost always developed in pars esophagea⁵², but the lesions observed in PFTS pigs were almost exclusively in the fundus. Further, the erosions described in mice and rats were grossly visible^{4,123,140}, but those in PFTS pigs were microscopic. Additionally, ulcerations were not a feature in PFTS pigs. Finally, although the attenuation of foveolar cells in PFTS pigs might represent disuse atrophy by the anorexia associate with PFTS, the increased lymphoplasmacytic infiltrate cannot be easily explained by anorexia alone. Taken all together, it is possible that the superficial gastritis may be the results of anorexia, but there is no clear evidence in the literature to support this hypothesis. It should be noted that in a previous study based on a small number of pigs, superficial gastritis was observed in healthy pigs on the day of weaning, in pigs fasted for 4 days and in pigs affected by PFTS.¹⁵⁴ This suggests that superficial gastritis is a background lesion present in healthy pigs around the time of weaning. It is however not known if the apparently healthy pigs at weaning with superficial gastritis experienced any degree of anorexia or reduced feed intake before sampling, nor was it possible to demonstrate prospectively that these pigs would not develop PFTS following weaning. The difficulty in separating potential causes of anorexia from effect of anorexia is innate to a case-control design chosen for this study. That being said, a case-control study was the only practical option to investigate the syndrome since the etiology is not known at this time. A fasting animal experiment is needed to answer important questions pertaining to the

occurrence of gastritis definitively.

Despite our poor understanding of the pathogenesis of the observed lesions, the high prevalence of superficial gastritis, small intestinal villous atrophy and thymic atrophy helps to characterize PFTS histologically and demonstrate that PFTS is a clinical syndrome with consistent pathology. Based on the present results, a presumptive histological diagnosis of PFTS can be made in cases in which stomach, small intestine and thymus are examined. A highly sensitive diagnostic criterion involves a pig/carcass having characteristic lesions present in at least two of three organs (i.e. having at least 2 or 3 of superficial gastritis, small intestinal villous atrophy or thymic atrophy). This criterion has a high sensitivity (100%) but moderate specificity (67.4%). Pigs/carcasses not satisfying this criterion can be classified as not having PFTS, but there is a high likelihood of false positive diagnoses. Alternatively, a moderately sensitive diagnostic criterion involves a pig/carcass having characteristic lesions present in all three organs. With this criterion, the false positive rate will decrease considerably, but one may misdiagnose some PFTS pigs as not having the syndrome (increased false negative rate). Therefore, we suggest this latter criterion be used when larger groups of animals are examined for herd level diagnosis. It should be noted that in farm 8 (a PFTS partial-farm), where only 3 CASE pigs but all 4 CTRL pigs had thymus, fundic stomach and small intestine for evaluation, all CASE pigs, regardless of which of the above criteria was used, were falsely classified as having PFTS, but all CTRL pigs were correctly identified as not having PFTS. This emphasizes that regardless of the histological criterion used, it is paramount that the diagnosis of PFTS is made only if the clinical case definition is fulfilled, characteristic lesions are present in GIT and thymus (as described above), and lesions and pathogens that can cause similar clinical presentation (e.g. pneumonia, polyserositis, PCVAD, PRRS, etc) are not present in the animals examined.

The results of the current study do not support a causal role for any of the pathogens investigated, which is consistent with previous reports^{76,154}. Collectively, the evidence to date demonstrates convincingly that PFTS is not caused by PCV2, PRRSV, FLUAV, RV-A and B, PECV, TGEV, Mhyo, Hh and Hp. RV-C is more prevalent in PFTS-farms than partial farms, which may indicate that RV-C is a risk factor for a farm to have PFTS. However, it should be noted that unlike RV-A and RV-B, the prevalence of RV-C was higher than 50% in all farms except farm 7. Thus, the impact of RV-C to nursery pig health is not clear. It is also unlikely that PFTS is caused by HEV and PEVs, since these viruses are detectable in both PFTS- and partial-farms, and in CASE and CTRL pigs with no difference in odds ratio.

Neither the presence of HEV nor PEVs was associated with the presence of lesions in the brain or trigeminal ganglion. Thus, the etiology of non-suppurative meningoencephalitis and trigeminal neuritis is not known. Furthermore, cytomegaloviral inclusion bodies were not consistently detected in affected farms. Thus, although HEV, PEV, and PMCV may contribute to the clinical signs of some PFTS-CASE pigs, they are clearly not a consistent and primary finding in all PFTS-farms.

Our present hypothesis is that the etiology of PFTS is consistent between farms. On the other hand, it cannot be ruled out that PFTS is caused by different etiologies on different farms, despite having consistent clinical presentation and lesions. It should be noted that this does not disqualify PFTS as a clinical syndrome. Clinical syndromes caused by different etiologies are not novel in swine medicine, for example, porcine respiratory disease complex. The pathogenesis of PFTS was not determined in this investigation. At the time of writing, the only peer-reviewed research on this topic showed that altered intestinal barrier function might be responsible for the development of PFTS, but the cause of this alteration was not clear.¹²⁵

In conclusion, PFTS-CASE pigs have significantly higher odds of exhibiting superficial gastritis, small intestinal villous atrophy and thymic atrophy compared to non-PFTS pigs. PFTS can be ruled out upon gross and histological examination if pigs/carcasses do not have at least 2 of these 3 lesions. PFTS should be suspected if necropsy cases have all three histological lesions, although ~15% false positive and false negative rates would be expected. Fulfillment of the clinical case definition, the presence of the above lesions and absence of underlying pathology and pathogens need to be considered together to diagnose PFTS at the farm level. The etiology of PFTS is not determined, but current evidence indicates it is not caused by any of the pathogens, including PCV2, PRRSV, FLUAV, RV-A and B, PECV, TGEV, Mhyo and others, investigated in this study. Finally, non-suppurative meningoencephalitis, neutrophilic lymphoplasmacytic rhinitis and RV-C infection may be risk factors for a farm to develop PFTS, but their potential roles in pathogenesis need further study.

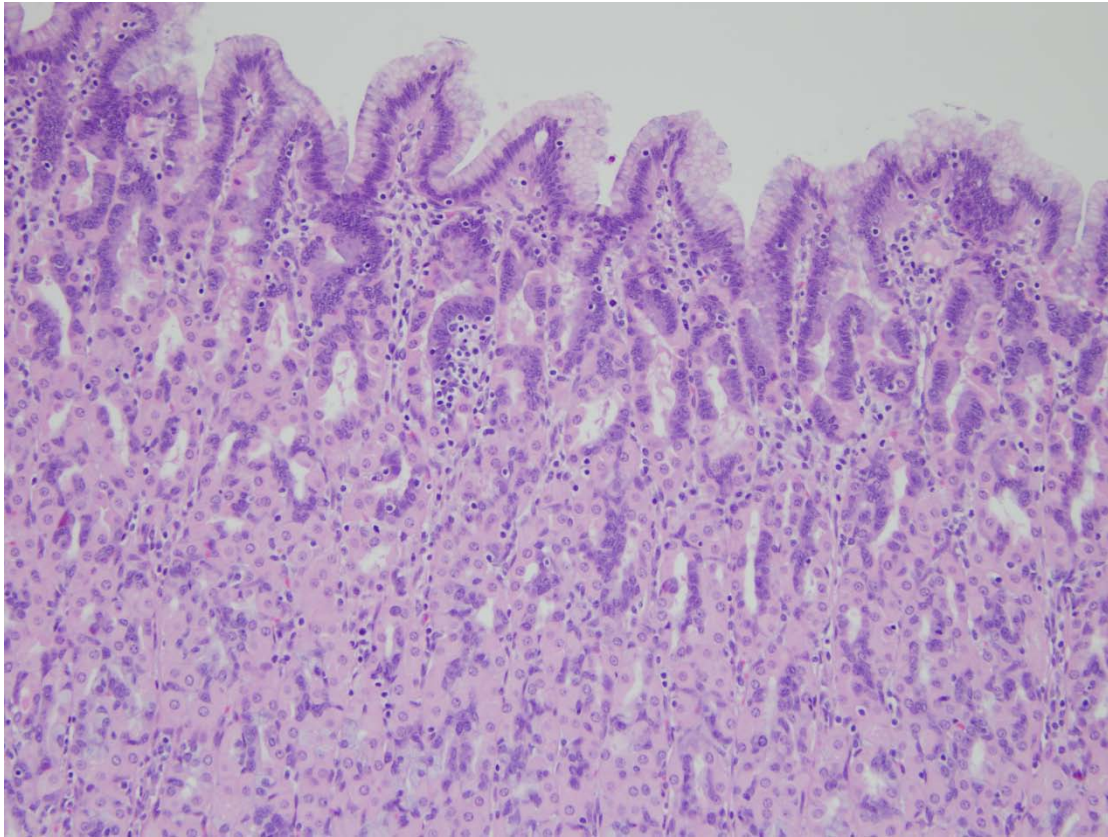


Figure 5.1. Gastric fundus; partial-CTRL pig. Note the abundant cytoplasmic mucus of foveolar cells.

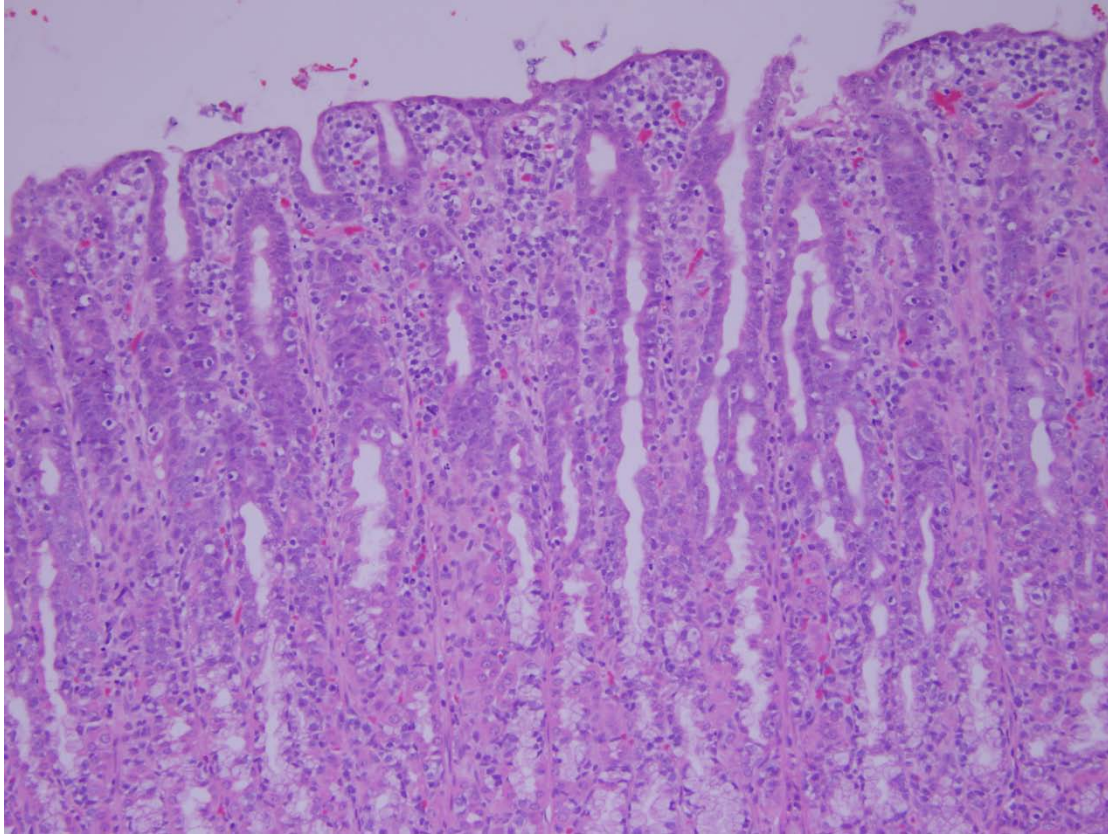


Figure 5.2. Gastric fundus; PFTS-CASE pig. Superficial foveolar cells lost cytoplasmic mucus and are attenuated. There is subtle increase of mononuclear cells underneath the foveolar cells.

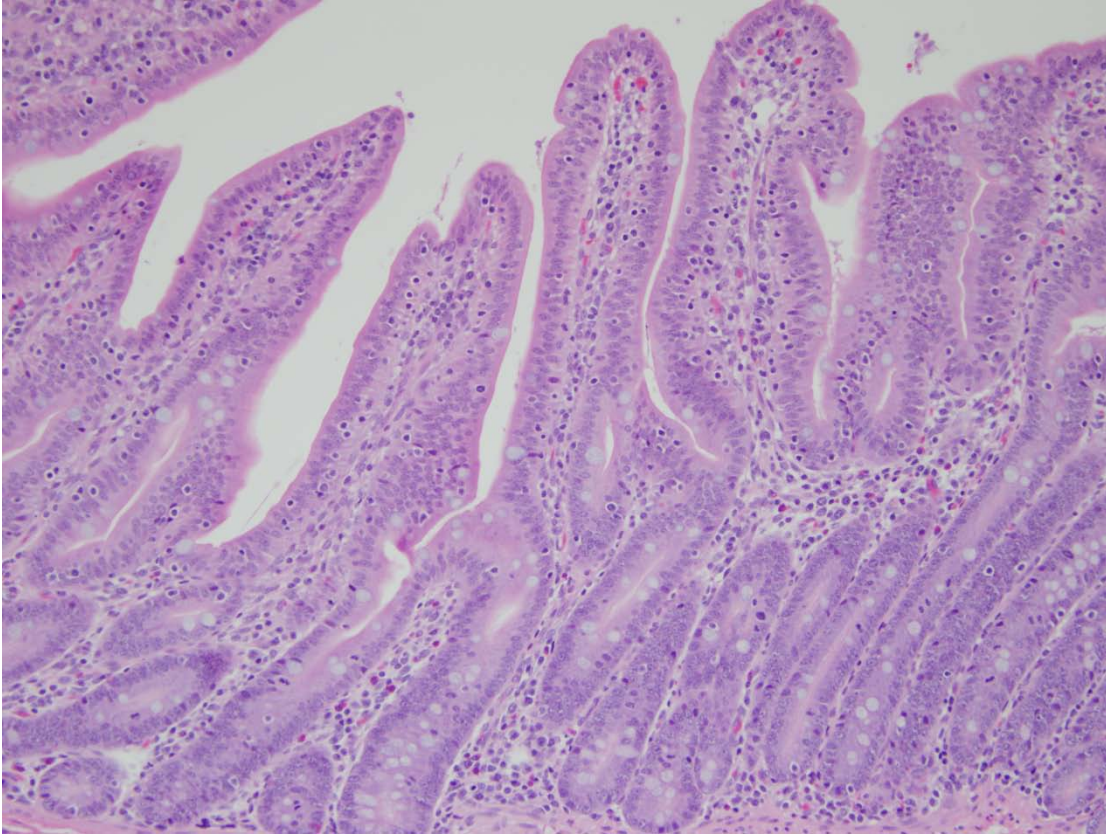


Figure 5.3. Jejunum; partial-CTRL pig. The villus to crypt ratio is slightly greater than 1.

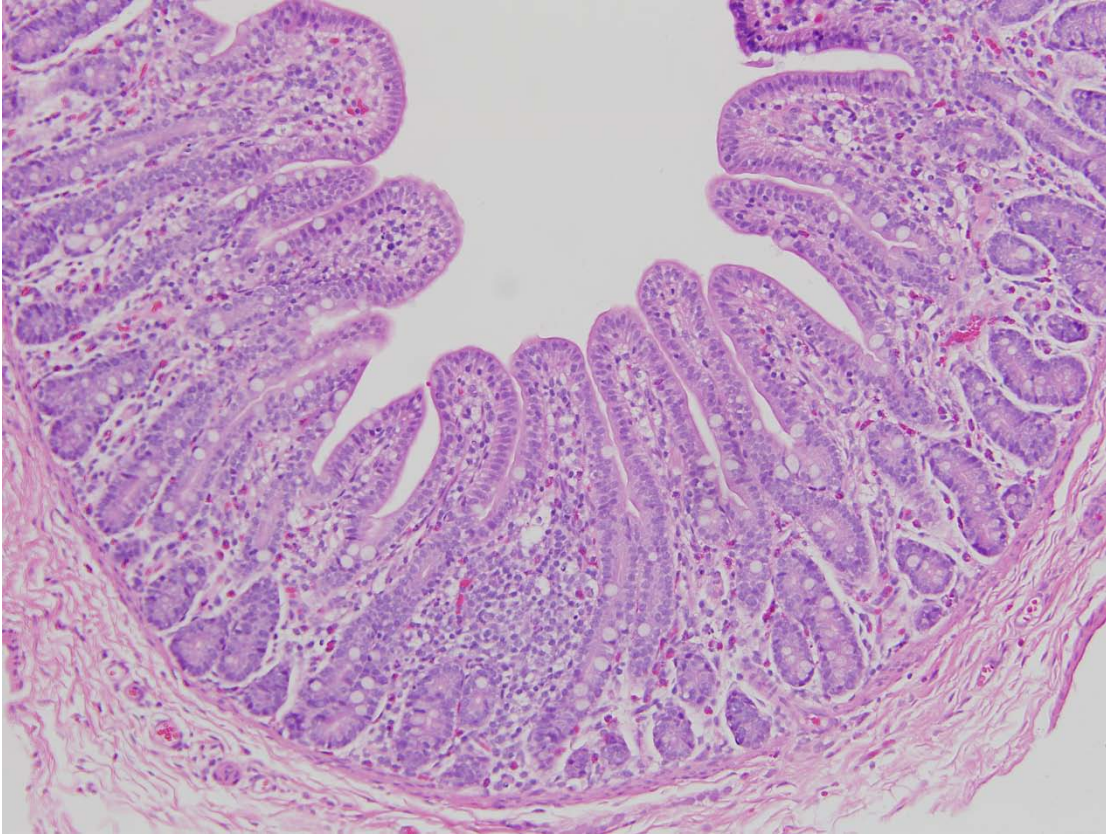


Figure 5.4. Jejunum; PFTS-CASE pig. The villi length and the total thickness are both reduced compared to that in Figure 5.3, which was taken at the same magnification.

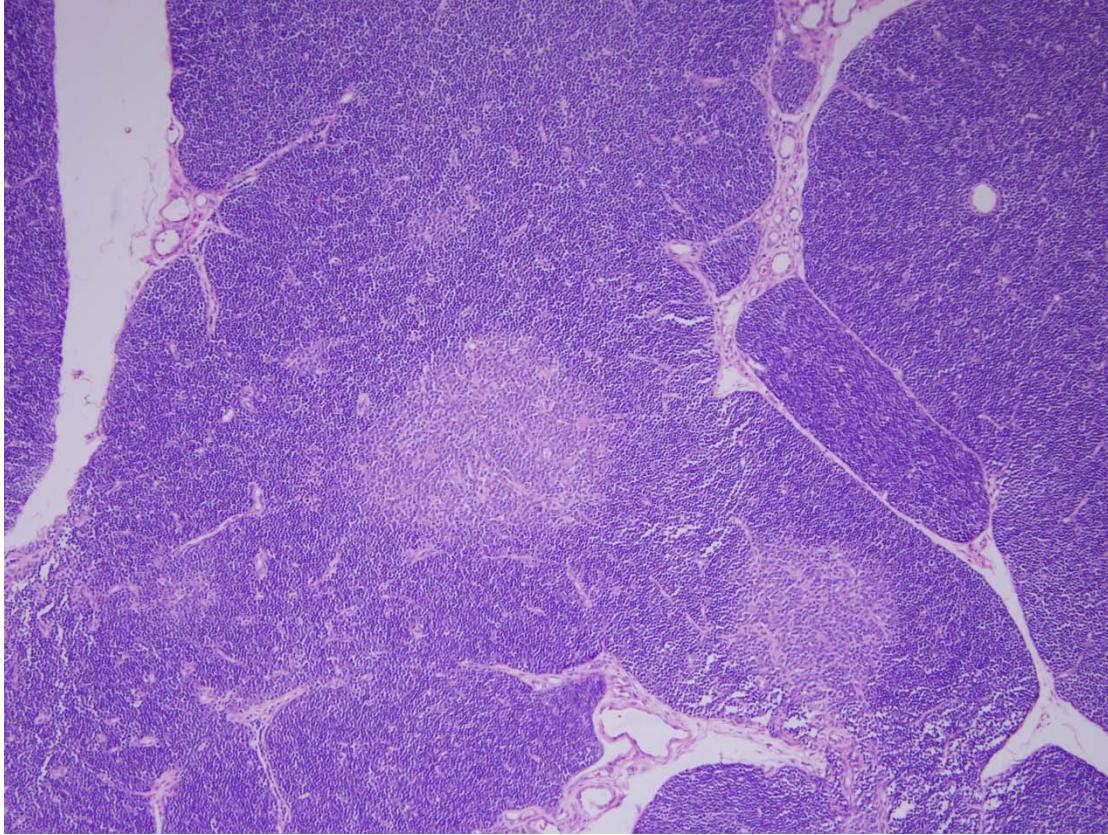


Figure 5.5. Thymus; partial-CTRL pig. Note the large numbers of lymphocytes in the cortex.

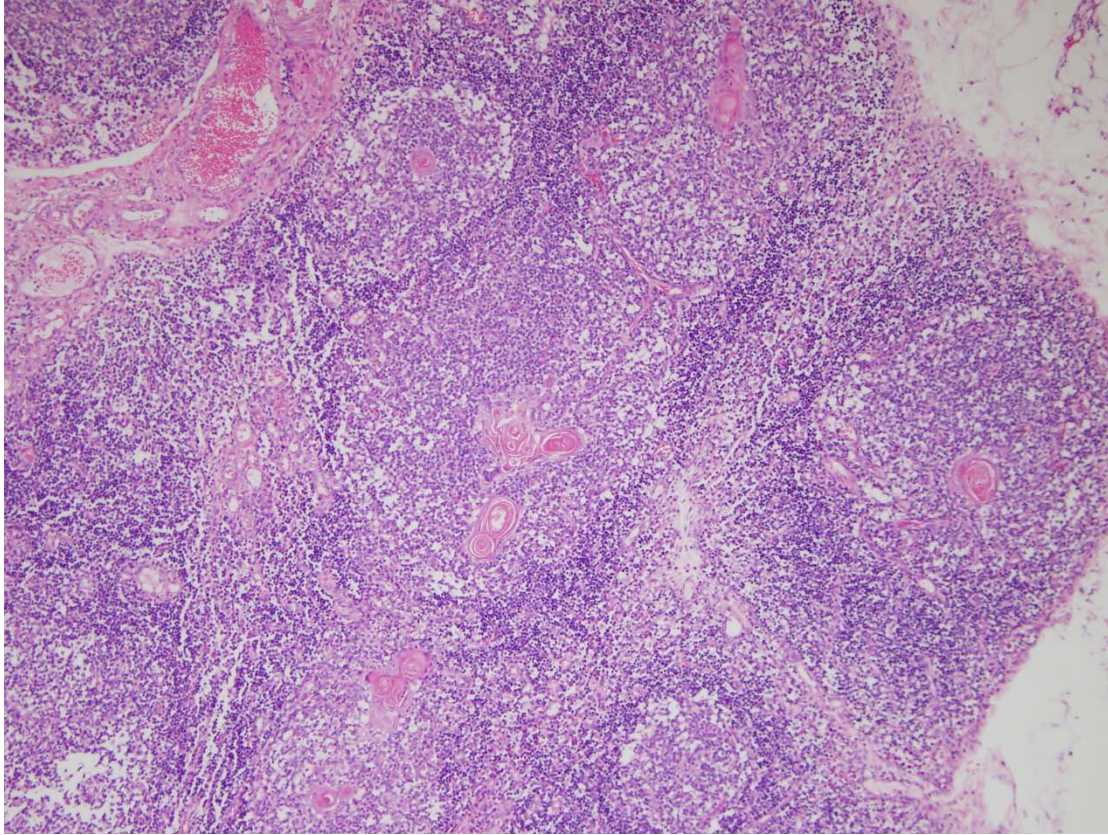


Figure 5.6. Thymus; PFTS-CASE pig. There is markedly reduced numbers of lymphocytes in the cortex.

Table 5.1. Historical details of farms included in the PFTS case-control diagnostic investigation

Farm	Location	Production type	Wean age	Brief clinical description of farm complaint	Pre-visit diagnostic results (screening)	Date of visit	Fulfill PFTS definition on farm visit?
PFTS-farms*							
1	Kansas, US	Nursery (1100 head)	D21	average nursery mortality 7.71% during outbreak of chomping and anorexia; 2.85% 6 months before outbreak	NA	July 2010	Completely
2	Kansas, US	Nursery (2100 head)	D21-24	up to 12.1% nursery mortality during outbreak of chomping and anorexia; average 3.1% 1 year before and 1 year after	NA	April 2011	Completely
3	Missouri, US	Nursery (2300 head)	D23	up to 5% batch-mortality during outbreak of chomping and anorexia; 2% in unaffected batches	No underlying pathology; group A rotavirus present in some pigs by PCR	April 2011	Completely
4	Alberta, Canada	Farrow-to-finish (450 sows)	D28	6-7% pigs described as “fading” noted within a week after weaning	No underlying pathology; no bacteria cultured responsible for clinical signs	February 2012	Completely
5	Alberta, Canada	Farrow-to-finish (480 sows)	D22	up to 10% nursery mortality during outbreak; baseline mortality 2.5%	No underlying pathology; no bacteria cultured responsible for clinical signs; influenza A and PCV2 PCR negative	February 2012	Completely
Partial-farms†							

Farm	Location	Production type	Wean age	Brief clinical description of farm complaint	Pre-visit diagnostic results (screening)	Date of visit	Fulfill PFTS definition on farm visit?
6	Ontario, Canada	Nursery (2400 head)	D17-21	20% nursery mortality when first contacted; historical mortality 2-4%	No significant pathology; PRRSV† and PCV2 IHC negative	April 2012	Partially (no progressive debilitation observed at visit)
7	Saskatchewan, Canada	Farrow-to-finish (660 sows)	D21	History of elevated numbers of fading, anorexic and chomping pigs; 2% nursery mortality at the time of visit	No significant pathology; no significant bacteria; Mhyo, PCV2 and influenza A PCR negative	October 2011	Partially (no progressive debilitation observed at visit)
8	Manitoba, Canada	3 sites 2000 head Nursery (2000 head)	D19-21	8% pigs put into hospital pen on visit, due to anorexia	No significant pathology; No significant bacteria; Mhyo, influenza A virus, PRRSV PCR negative	October 2011	Partially (all case pigs affected by bronchopneumonia)

* PFTS-farms: Farms completely fit PFTS clinical case definition⁸ on farm visit;

† Partial-farms: Farms partially fit PFTS clinical case definition on farm visit;

‡ Legend: PRRSV=porcine reproductive and respiratory syndrome virus, PCV2=porcine circovirus type 2, IHC=immunohistochemistry, Mhyo=*Mycoplasma hyopneumoniae*

Table 5.2. Prevalence and odds ratios indicating significance of histopathological lesions observed in PFTS case and control pigs

	PFTS-farms*		Partial-farms†		P values			Odds ratio (95% CI)	
	Case (%)	Control (%)	Case (%)	Control (%)	Farm -type‡	Pig -type§	Inter-action	PFTS-CASE vs. all other	PFTS-farms vs. partial-farms
Superficial gastritis	33/36** (91.7) ^{a††}	9/20 (45.0) ^b	10/22 (45.5) ^b	3/11 (27.3) ^b	<0.0001	<0.0001	0.038	16.7 (6.8-40.6)	NA‡‡
Duodenal villous atrophy	35/36 (97.2) ^a	6/18 (33.3) ^b	11/18 (61.1) ^{ab}	3/7 (42.9) ^b	<0.0001	<0.0001	0.01	28.7 (7.7-107)	NA
Jejunal villous atrophy	34/35 (97.1) ^a	6/17 (33.3) ^b	7/18 (38.9) ^b	2/8 (25.0) ^b	<0.0001	0.002	0.026	67.4 (7.0-651)	NA
Ileal villous atrophy	35/37a (94.6) ^a	5/19 (26.3) ^b	7/23 (30.4) ^b	2/11 (18.2) ^b	<0.0001	0.001	0.012	56.3 (14.1-224)	NA
Thymic atrophy	27/31 (87.1) ^a	3/19 (15.8) ^b	5/18 (27.8) ^b	1/12 (8.3) ^b	<0.0001	0.001	0.257	30.1 (6.0-152)	NA
Rhinitis	26/38 (72.2)	17/20 (85.0)	11/24 (45.8)	6/12 (50.0)	0.037	0.159	0.36	NA	3.7 (1.1-12.5)
Encephalitis or trigeminitis	25/40 (62.5) ^a	11/20 (55.0) ^a	7/25 (28.0) ^b	2/12 (16.7) ^c	0.014	<0.0001	0.16	NA	4.7 (1.4-15.9)
Colitis	10/32 (31.3)	1/17 (5.9)	0/17 (0.0)	0/8 (0.0)	NA	NA	NA	14.6 (2.0-107)	NA
Broncho pneumonia	11/40 (27.5)	2/20 (10.0)	11/25 (44.0)	4/12 (33.3)	0.145	0.33	0.640	NA	NA
Hepatic fatty change	6/40 (15.0)	0/20 (0.0)	0/17 (0.0)	0/8 (0.0)	NA	NA	NA	NA	NA
Lymphocytic nephritis	4/40 (10.0)	6/19 (31.6)	1/17 (5.9)	0/8 (0.0)	NA	NA	NA	NA	NA

*PFTS-farms: Farms completely fulfill the PFTS clinical case definition at farm visit;

†Partial-farms: Farms partially fulfill the PFTS clinical case definition at farm visit;

‡Farm-type: PFTS- or partial farms;

§Pig-type: case or control pigs;

||PFTS-case pigs: case pigs from PFTS-farms;

¶The initial GEE analysis was not possible for colitis because of its absence in some groups. However, it was possible to compute an odds ratio between case pigs in PFTS-farms and all other pigs;

**Values in bold font if lesion prevalence is >60%. The denominators of the prevalence are not consistent between different tissues because some tissues failed to be collected for histological evaluation;

††In each row, different superscript letters represent difference of post hoc pairwise comparisons;

‡‡NA= Not applicable.

Table 5.3. Villous lengths, crypt depths and villi to crypt (V/C) ratios measured in case and control pigs in this study

	PFTS-farms*		Partial-farms†		P values		
	Case (±SD)	Control (±SD)	Case (±SD)	Control (±SD)	Pig-type‡	Farm-type§	Interaction
Villous lengths (µm)	163±81 ^a	330±89 ^b	327±116 ^b	385±76 ^b	<0.0001	<0.0001	0.008
Crypt depths (µm)	184±32 ^a	213±30 ^b	188±64 ^a	181±37 ^a	0.232	0.021	<0.0001
V/C ratios	0.9±0.4 ^a	1.6±0.5 ^b	1.9±1.0 ^c	2.2±0.7 ^c	<0.0001	<0.0001	0.062

*PFTS-farms: Farms completely fulfill the PFTS clinical case definition at farm visit;

†Partial-farms: Farms partially fulfill the PFTS clinical case definition at farm visit;

‡Farm-type: PFTS- or partial farms;

§Pig-type: case or control pigs;

Within each row, different superscript letters represent differences of post hoc pairwise comparisons

Table 5.4. Comparative assessment of the presence of ileal villous atrophy using microscopic estimation and exact measurements

Villous atrophy (Yes/No)		Using exact V/C ratio* less or equal to 1 as gold standard		Sensitivity (%;95% CI)	Specificity (%;95% CI)
		Yes	No		
Microscopic estimation	Yes	44	5	100 (92-100)	89 (77-95)
	No	0	41		
Using exact V/C ratio less or equal to 1 as gold standard					
		Yes	No		
Microscopic estimation	Yes	46	3	100 (92-100)	93 (82-98)
	No	0	41		

*V/C ratio = villi to crypt ratio

Microscopic estimation: When the general villi to crypt ratio was estimated to be equal or less than 1, villous atrophy was assigned.

Table 5.5. Prevalence, sensitivity, specificity and predicted values of accurately diagnosing PFTS cases using at least two or all three characteristic lesions: superficial gastritis, small intestinal villous atrophy and thymic atrophy

	PFTS-farms*		Partial-farms†		All non-PFTS pigs‡ (%)	Sensitivity (%; 95%CI)	Specificity (%; 95%CI)	Positive predictive value (%; 95%CI)	Negative predictive value (%; 95%CI)
	Case (%)	Control (%)	Case (%)	Control (%)					
Having at least 2 lesions	29/29 (100)	6/18 (30)	6/15 (40)	2/10 (20)	14/43 (32.6)	100 (88-100)	67.4 (53-80)	67.4 (53-80)	100 (88-100)
Having all 3 lesions	24/28 (85.7)	2/18 (11.1)	4/18 (22.2)	1/10 (10)	7/46 (15.2)	85.7 (69-96)	84.8 (65-90)	77.4 (60-89)	90.7 (73-95)

*PFTS-farms: Farms completely fulfill PFTS clinical case definition on farm visit;

†Partial-farms: Farms partially fulfill PFTS clinical case definition on farm visit;

‡Non-PFTS pigs: All pigs in this study except case pigs from PFTS-farms

Table 5.6. Prevalence of common swine pathogens in case and control pigs from each farm included in the investigation

Pathogens (organ)	Farm	Prevalence (%)							
		1	2	3	4	5	6	7	8
RV-A* (Ileum)	Case	4/8 (50)	2/8 (25)	6/8 (75)	1/8 (12.5)	0/8 (0)	4/8 (50)	4/9 (44.4)	5/8 (62.5)
	Control	1/4 (25)	3/3 (100)	4/4 (100)	0/4 (0)	1/4 (25)	3/4 (75)	2/4 (50)	3/4 (75)
RV-B* (Ileum)	Case	6/8 (62.5)	1/8 (12.5)	3/8 (37.5)	3/8 (37.5)	1/8 (12.5)	2/8 (25)	2/9 (22.2)	2/8 (25)
	Control	3/4 (75)	0/3 (0)	2/4 (50)	1/4 (25)	1/4 (25)	2/4 (50)	1/4 (25)	4/4 (100)
RV-C* (Ileum)	Case	7/8 (87.5)	8/8 (100)	5/8 (62.5)	7/8 (87.5)	6/8 (75)	5/8 (62.5)	3/9 (33.3)	6/8 (75)
	Control	4/4 (100)	2/3 (66.7)	2/4 (50)	3/4 (75)	3/4 (75)	3/4 (75)	1/4 (25)	2/4 (50)
HEV (Tonsil)	Case	0/8 (0)	3/8 (37.5)	3/8 (37.5)	1/8 (12.5)	0/8 (0)	5/8 (62.5)	1/9 (11.1)	0/8 (0)
	Control	1/4 (25)	1/4 (25)	3/4 (75)	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)	1/4 (25)
PEV1	Case	4/8	5/8	0/8	0/8	0/8	1/8	9/9	0/8

Pathogens (organ)	Farm	Prevalence (%)							
		1	2	3	4	5	6	7	8
(Brain)		(50)	(62.5)	(0)	(0)	(0)	(12.5)	(100)	(0)
	Control	2/4	2/4	1/4	1/4	0/4	0/4	3/4	1/4
PEV2		(50)	(50)	(25)	(25)	(0)	(0)	(75)	(25)
	Case	8/8	6/8	2/8	0/8	4/8	1/8	4/9	0/8
(Brain)		(100)	(75)	(25)	(0)	(50)	(12.5)	(44.4)	(0)
	Control	4/4	2/4	1/4	1/4	0/4	0/4	0/4	1/4
PEV3		(100)	(50)	(25)	(25)	(0)	(0)	(0)	(25)
	Case	8/8	7/8	2/8	0/8	2/8	6/8	1/9	1/8
(Brain)		(100)	(87.5)	(25)	(0)	(25)	(75)	(11.1)	(12.5)
	Control	2/4	2/4	3/4	0/4	0/4	3/4	0/4	2/4
		(50)	(50)	(75)	(0)	(0)	(75)	(0)	(50)

* Legend: RV-A = group A rotavirus; RV-B = group B rotavirus; RV-C = group C rotavirus; HEV = haemagglutinating encephalomyelitis virus; PEV1, 2 and 3 = porcine enteroviruses CPE group 1, 2 and 3

One PFTS-CASE (from farm 1) was positive for porcine type 2 circoviruse (PCV2, tonsil), and all pigs were negative for porcine reproductive and respiratory syndrome virus (PRRSV, lung), influenza A virus (FLUAV, lung), porcine enteric calicivirus (PECV, ileum), transmissible gastroenteritis virus (TGEV, ileum), *Mycoplasma hyopneumoniae* (Mhyo, lung), *Helicobacter-pylori*-like (Hp, stomach) and *Helicobacter-heilmannii*-like (Hh, stomach) organisms.

6. Clinical pathology, serum cytokines and Vitamin D changes associated with Porcine Periweaning Failure to Thrive Syndrome (PFTS)

Chapters 4 and 5 identified several consistent lesions of PFTS. However, no antemortem blood parameters have been thoroughly evaluated. Yet these may provide important insights in the diagnosis and pathogenesis of PFTS. Thus, in Chapter 6, clinical pathology and serum cytokines were characterized in PFTS pigs and compared to non-affected pigs.

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Huang, Y. and J.C.S. Harding, Clinical pathology, serum cytokines and Vitamin D changes associated with Porcine Periweaning Failure to Thrive Syndrome (PFTS). BMC Vet Res, 2013. Manuscript in preparation.

6.1. Introduction

Porcine periweaning failure-to-thrive syndrome (PFTS) is characterized clinically by anorexia and progressive debilitation of newly weaned pigs. Up to 20% nursery pigs within 2-3 weeks of weaning can be affected.⁷⁹ A sporadic condition clinically similar to PFTS at individual pig level but of lower morbidity and mortality was usually referred to as “post-weaning starve-outs” and may have existed for decades. However, in 2008 outbreaks of “starve-outs” which caused severe nursery mortality were recognized as a distinct clinical syndrome in North America.^{42,57} It has been estimated that 4.3% of nursery flows in North America were affected, and the within-flow prevalence of PFTS was most commonly between 1-10%.¹³⁹ It is not known whether PFTS is present in other continents, but a recent report suggests it may be present in Spain.¹⁷⁸

The etiology of PFTS is unknown, but a number of swine pathogens have been ruled out including porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, influenza A virus, transmissible gastroenteritis virus, *Mycoplasma hyopneumoniae*, *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, *Helicobacter-pylori*-like and *Helicobacter-heilmanii*-like organisms.^{76,78,154} Additionally, porcine cytomegalovirus, porcine enteric calicivirus, swine Torque Teno virus 2, pathogenic *Escherichia coli*, rotavirus, haemagglutinating encephalomyelitis virus, porcine enterovirus CPE groups 1, 2, 3 and coccidia have been detected in some affected pigs as well as in age-matched unaffected pigs and the prevalence did not differ.^{76,154} Thus, these pathogens are not likely to be the etiology of PFTS.

The diagnosis of PFTS is based on fulfillment of the current case definition⁷⁹ and exclusion of other common swine diseases. The PFTS case definition is clinically oriented and was developed in 2010 prior to understanding the pathological features of the syndrome. It is as follows: *PFTS*

*is characterized by anorexia and progressive debilitation of newly weaned pigs with variable morbidity but high case fatality, with repetitive oral behaviors such as chomping and chewing on farm level, without residual illness from the suckling phase, and without discernible and detrimental infectious, nutritional, managerial and environmental factors that can explain the clinical symptoms.*⁷⁹ Recently, the authors have completed a case-control pathological investigation of PFTS affected and non-affected pigs from a number of farms across North America. Additional criteria for the diagnosis of individual animals were proposed which included 1) satisfying the PFTS clinical definition, 2) the presence of thymic atrophy, superficial gastritis and small intestinal villous atrophy, and 3) ruling out of all other relevant diseases and porcine pathogens potentially affecting young nursery pigs.⁷⁸

Clinical pathology may help to elucidate the pathophysiological mechanism(s) associated with PFTS, and may also direct future etiological and epidemiological research. At the time of writing, such data has only been reported in one study investigating one affected farm with limited sample size.¹⁵³ In another study, unlike age-matched fasted pigs, PFTS pigs failed to exhibit elevated urine ketone bodies suggesting an abnormal metabolic response underlying PFTS.¹²⁵ These results however, need to be verified with a larger, multi-farm study.

It has been conjectured that the progressive debilitation of PFTS may be associated with a systemic proinflammatory cytokine response.^{42,125} This hypothesis was supported in porcine postweaning multisystemic failure syndrome (PMWS),⁹³ but data is not available for PFTS. Finally, concurrent with the recognition of PFTS in North America, some regions reported universal vitamin D deficiency in nursery pigs.¹⁹⁹ The lack of case-control or experimental data led to our interest in further clarifying the relationship between serum vitamin D levels and PFTS. To this end, the objectives of this study are to compare haematology, serum chemistry, cytokine

and vitamin D levels in PFTS-affected and age-matched control pigs with the intent of elucidating any underlying pathophysiological mechanisms associated with the development of PFTS.

6.2. Methods

6.2.1. Farm visits and sample collection

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Eight (8) or 9 CASE and 4 CTRL pigs blocked by days postweaning were selected in each of 8 farms which allegedly fit the PFTS clinical definition identified during a diagnostic investigation as described previously (Chapter 5). Blood samples were collected from the cranial vena cava vein into EDTA and plain (non-anticoagulated) tubes for subsequent analyses.

6.2.2. Categorization of farms based on clinical presentation

As described in Chapter 5, 5 farms (farm 1-5) completely fit the PFTS clinical definition⁷⁹ but farm 6, 7 and 8 only partially fulfilled this definition. Farms 1-5 were thus designated as "PFTS" farms and 6-8 as "partial" farms. As a result, there were 4 subgroups in this study: 1) affected pigs from PFTS-farms (PFTS-CASE); 2) non-affected pigs from PFTS-farms (PFTS-CTRL); 3) affected pigs from partial-farm (partial-CASE); and 4) non-affected pigs from partial-farm (partial-CTRL). The partial-farms were included in the analysis because they might be useful for comparison.

6.2.3. Haematology and serum chemistry

Approximately 5 ml of EDTA blood from each pig on all farms except farms 1 and 6 was submitted to Prairie Diagnostic Services (PDS) Inc. (Saskatoon, SK, Canada) for complete blood count (CBC) using a Celldyn 3500R Automated Haematology Analyzer (Abbott Laboratories, Abbott Park, Illinois, USA). CBC for samples from farm 6 was performed at the Animal Health Laboratory (AHL), University of Guelph using an ADVIA 210i automated haematology analyzer (Siemens Healthcare Diagnostics Ltd., Mississauga, ON, Canada). Differential counts were performed by manually counting 100 cells. A CBC was not performed for farm 1 pigs. Serum chemistry was performed at PDS Inc. using Cobas c311 analyzer (Roche Diagnostics, Mannheim, Germany). Beta hydroxybutyrate (BHB) levels were tested using a Ranbut reagent kit (Randox Laboratories Limited, County Antrim, UK). Results were compared to previously described reference values of “weaner pigs”.⁵³ The above tests were not performed in samples from farm 1.

6.2.4. Serum cytokine concentrations

Sera were obtained by centrifugation of approximately 10 ml non-anticoagulated blood and stored at -80°C before testing for IL-1 β , IL-4, IFN- α , IL-10, IL-12, CCL2 and IL-8 using a 7-plex fluorescent microbead immunoassay (FMIA) as previously described¹⁰³ with minor modifications. TNF α and IFN- γ were not included in the panel, and CCL2 monoclonal antibody was provided by Dr. Joan Lunny, Beltsville Agricultural Research Center (Beltsville, MD USA). Standards and samples were tested in duplicate, and the observed concentrations of standards were within 20% of expected concentrations. The dynamic ranges of each analyte were: IL-1 β : 8-5000 pg/ml; IL-4: 3-2000 pg/ml; IFN- α : 3-5000 pg/ml; IL-10: 3-2000 pg/ml; IL-12: 20-5000

pg/ml; CCL2: 50-5000 pg/ml and IL-8: 20-2000 pg/ml. Three 96 well plates were used to test samples. Duplicates exhibiting more than 30% coefficient of variation (CV) were re-tested. Aliquots of quality control sera which contained known concentrations of each analyte were included on each plate. All quality control samples had CV less than 10%.

Serum IFN- γ concentration was tested using the Swine IFN-g Antibody Pair ELISA kit (Life Technology Inc. Burlington, ON, Canada) according to the manufacturer's instructions, except that the color substrate employed was KPL SureBlue Reserve TMB Microwell Substrate (Mandel Scientific Company Inc., Guelph, ON, Canada). After adding color substrate, the plates were incubated in the dark for 25 minutes before the absorbance of each well was measured at 650 nm on a Vmax microplate reader (Molecular Devices, LLC., Sunnyvale, CA, USA). All sera were diluted 1:3 in negative sera and tested in duplicate.

6.2.5. Serum vitamin D

Sera 25-OH vitamin D assay was performed by Heartland Assays, LLC (Ames, Iowa, USA) using a two-step procedure.⁷⁴ The first involved a rapid extraction of 25-OH vitamin D and other hydroxylated metabolites from sera with acetonitrile. Following extraction, the treated samples were then assayed using an equilibrium radial immunoassay (RIA) procedure. The RIA method is based on an antibody that is co-specific for 25-OH vitamin D₂ and 25-OH vitamin D₃. The samples, antibody and tracer were incubated for 120 minutes at 20-25°C. Phase separation was accomplished after a 20 minute incubation at 20-25°C with a second antibody precipitating complex. A non-specific binding (NSB)/addition buffer was added after this incubation prior to centrifugation to aid in reducing non-specific binding. 25-OH vitamin D equivalent values were calculated directly by the γ -radiation counting system with use of a smooth-spline method. The

results were expressed in terms of 25-OH vitamin D equivalents. The assay has a range of 2.5-100 ng/ml and intra- and inter-assay CV of 8.0% and 10.0% respectively.

6.2.6. Statistical analyses

Generalized Estimating Equations (GEE) accounting for clustering by farm were used to build linear regression models, in which the outcomes were parameters of serum chemistry or haematology and the predictors farm-type (PFTS-farms or partial-farms), pig-type (CASE or CTRL) and the interaction term of these two factors. All models used an exchangeable correlation matrix with a robust variance estimator. The normality and homoscedasticity of the raw residuals of each final model were evaluated. In models where the assumptions of normality or homoscedasticity were violated, the specific parameters were transformed (natural log [ln], log₁₀ or square root) and re-analyzed. Pairwise post hoc comparisons of the estimated means of each combination of farm-type and pig-type were performed when the interaction term was statistically significant, or when both farm-type and pig-type were significant. If the transformed models still violated the above two assumptions, the data were then categorized, and a binary or ordinal logistic regression by GEE was used to re-analyze the data. In the case of dichotomized data that could not be analysed using GEE, a Fisher's exact test was used. All statistical analyses were performed using IBM SPSS Statistics version 21 (IBM Corp., New York). To reduce the risk of type 1 error associated with testing multiple potentially related parameters in the same samples, *P* values less than 0.01 were regarded as statistically significant.

6.3. Results

The results are presented in three tables: Table 6.1 for parameters in which the interaction terms of farm-type and pig-type were significant (except for urea which is also included in this table);

Table 6.2 in which only pig-type was significant, and Table 6.3 in which data was dichotomized.

Compared to all other pigs, PFTS-CASE pigs had significantly higher red blood cell count (RBC), hemoglobin concentration (Hgb), haematocrit (Hct), mean corpuscular hemoglobin concentration (MCHC), urea, and total, direct and indirect bilirubin than CTRL ($P < 0.0001$ for all) (Table 6.1). The predicted Hct, total and indirect bilirubin levels of PFTS-CASE pigs were higher than the reference ranges.⁵³ The predicted RBC, Hgb, urea, direct bilirubin, and albumin of all groups were within the reference ranges.⁵³ The predicted MCHC of all groups were lower than the reference range (Table 6.1).⁵³

Calcium, phosphorous, glucose, aspartate transaminase (AST) and vitamin D levels were significantly lower in CASE versus CTRL pigs regardless of farm-type ($P < 0.01$ for all) (Table 6.2). The predicted calcium, phosphorus, glucose and AST were within reference ranges.⁵³ Serum vitamin D concentrations of both CASE and CTRL pigs were lower than the published values (Table 6.2).²

The values for glutamate dehydrogenase (GLDH) and beta hydroxybutyrate (BHB) were dichotomized and analyzed by Fisher's exact test. The negative cut-offs for GLDH and BHB were 2U/L and 0.1mmol/L, respectively. PFTS-CASE pigs were compared to all other pigs. Half (20/40, 50%) of PFTS-CASE pigs had serum GLDH higher than the cut-off, compared to zero in any other group. Most (29/40, 76.3%) PFTS-CASE pigs had serum BHB higher than the cut-off, compared to only 4/57 (7%) non PFTS-CASE pig. Both variables were significantly higher in PFTS-CASE pigs than CTRL ($P < 0.0001$; Table 3).

There were no significant group differences for total white blood cells, eosinophils, basophils, monocytes, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), sodium,

potassium, chloride, bicarbonate, anion gap, magnesium, creatinine, gamma-glutamyltransferase (GGT), creatine kinase (CK), total protein or globulin.

The pig-type*farm-type interaction terms were not significant in all GEE models used to analyze serum cytokines. Pig-type significantly predicted serum IL-12 concentration, with CASE pigs having higher predicted levels (110.4 ± 21.8 pg/ml) than that CTRL pigs (36.7 ± 9.7 pg/ml), regardless of farm-type. Farm-type and pig-type were not associated with the serum concentrations of IL-1 β , IL-4, IL-10, CCL2, IL-8, IFN- α or IFN- γ (data not shown).

6.4. Discussion

The results of this study revealed significant haematological and serum biochemical differences in pigs affected by PFTS compared to controls, including higher levels of RBC, Hgb, Hct, MCHC, urea, bilirubin, albumin, AST, GLDH and BHB, as well as lower levels of calcium, phosphorus, glucose and vitamin D. Although there are a number of differential diagnoses for the observed differences in each individual analyte, reduced feed and water intake are the most likely cause of all of these differences.

The higher levels of RBC, Hgb, Hct, MCHC are consistent with erythrocytosis. These haematological parameters, which collectively reflect the quantities of RBC and hemoglobin, are closely related mathematically and their values usually move in the same direction as observed in the current study. The most common causes for their increase are hemoconcentration (i.e. dehydration) and splenic contraction in response to excitement or fighting associated with epinephrine release.¹⁸⁸ Whether or not splenic contraction was present in the PFTS-CASE pigs was not evaluated, but these pigs appeared depressed rather than excited, therefore erythrocytosis due to splenic contraction is unlikely. Conditions causing sustained hypoxia, such as pulmonary

disease and congenital cardiac vascular shunts (right to left), can also cause erythrocytosis due to increased bone marrow RBC production in response to increased renal erythropoietin production.¹⁸⁸ However, pneumonia was not a consistent feature of PFTS-CASE pigs and cardiac shunts were not observed.

The haematological findings in the current study agreed with those found in other studies investigating clinical pathology of PFTS and of unthrifty nursery pigs. Pittman and Moeser investigated pigs on the day of weaning, fasted pigs 4 days post-weaning, PFTS pigs 4 and 11 days postweaning, and control pigs 4 and 11 days post-weaning.¹⁵³ Pack cell volume (PCV) was significantly higher in PFTS and fasted compared to control pigs. Pittman's finding confirmed that 4-day fasting can induce increased PCV to the same magnitude as PFTS does in nursery pigs. Thus it is reasonable to conclude that the differences in PCV/Hct in PFTS pigs in the present study were likely due to anorexia. Buzzard *et al.* investigated blood parameters in unthrifty nursery pigs (n=32) compared to healthy controls (n=26).¹⁹ They reported significantly higher Hct and Hgb in the unthrifty pigs and concluded that these were indicative of dehydration and anorexia. Furthermore, Buzzard implied that the so-called unthrifty pigs could represent early stages of PFTS,¹⁹ but the authors of the present study remain neutral regarding Buzzard's supposition about the relationship between unthrifty and PFTS pigs.

The elevated blood urea in PFTS-CASE pigs also suggests anorexia or dehydration in PFTS pigs. Urea is converted from tissue or intestinal ammonium (NH₄⁺) in hepatocytes. Increased serum urea can be the result of reduced urinary excretion by a pre-renal process such as dehydration or shock, renal diseases, or urinary tract obstruction.¹⁸⁸ There was no evidence of shock, renal and urinary tract diseases in PFTS-CASE pigs. Increased urea production due to intestinal hemorrhage or increased protein catabolism can also account for increased blood urea. No

intestinal hemorrhage was observed in the current investigation, however increased protein catabolism is possible considering PFTS-CASE pigs experienced anorexia and alternative energy sources (e.g. fat and muscle mass) would be required for maintenance. Thus, the elevated blood urea was likely due to the combined effect of dehydration, anorexia and protein catabolism. However, it should be noted that urea concentration in PFTS-CASE pigs remained within the normal reference range, thus, it was more likely a physiological adaptation than pathological event. In the Pittman study, blood urea levels in PFTS and fasted pigs were higher than those in control pigs, but were not statistically different between groups.¹⁵³ Blood urea was not measured in the Buzzard study.¹⁹

In anorexia, hepatocellular uptake of bilirubin decreases, followed by an increase in indirect bilirubin in blood.¹⁸⁸ Other causes of hyperbilirubinemia include hemolysis, primary hepatic disease and cholestasis. As there was no evidence of overt hemolysis or hepatic pathology in the current investigation, the elevated total bilirubin (mainly due to indirect (unconjugated) bilirubin over the reference range) was most likely the result of anorexia. Although the patency of bile ducts was not examined in this study, hyperbilirubinemia caused by cholestasis is usually dominated by direct bilirubin, which was not the case in PFTS-CASE pigs. Consistent with our interpretation, the fasted pigs in the Pittman study demonstrated hyperbilirubinemia.¹⁵⁴ However, whether this was due to indirect bilirubin was not reported.

Elevated serum albumin in pigs is almost always the result of dehydration and no compelling differential diagnoses for these results in PFTS-CASE pigs were noted.¹⁸⁸

The high BHB levels in a large proportion of PFTS-CASE pigs indicate increased fat metabolism. Ketone bodies include acetone, acetoacetate and BHB, with BHB being most stable.¹⁸⁸ Thus, the

measurement of BHB should most reliably reflect the ketone status of an animal. Anorexia and diabetes mellitus are two of the most common reasons for increased BHB in the blood.¹⁸⁸ In the absence of pancreatic lesions, diabetes mellitus is unlikely, and anorexia is the most reasonable explanation for the increased BHB observed in this study. The BHB reference value for pigs is not available to the best of the authors' knowledge. But as a general rule, blood BHB concentration of greater than 1.4 mmol/L is reflective of ketosis in animals.¹⁸⁸ Accordingly, none of the pigs in the current study should be diagnosed as ketotic and the elevated BHB likely reflects anorexia but may not have contributed significantly to the clinical sickness. In Pittman and Moeser's clinical pathological investigation of PFTS and fasted pigs, fasted pigs exhibited ketonuria, but PFTS pigs did not.^{125,153} It was suggested that PFTS pigs might have a unique metabolic disorder.¹²⁵ The Pittman study however, did not measure blood ketone levels, and the specific ketone metabolite measured in urine was not specified.¹⁵³

The homeostasis of serum calcium and phosphorus are tightly connected and regulated by vitamin D. The major source of calcium, phosphorus and vitamin D for commercial pigs raised indoors is their diet.^{188,199} The current study revealed that the serum calcium, phosphorus and vitamin D levels were significantly lower in CASE than in CTRL pigs. There has been a resurgence of research interest in vitamin D in pigs because it has been recognized that nursery pigs in modern swine production systems have a high prevalence of hypovitaminosis D.¹⁹⁹ Although this finding was confirmed in this study, none of the pigs in this study demonstrated lesions of rickets. Since none of the pigs were exposed to sunlight, reduced feed intake was likely responsible for the lower serum vitamin D levels in CASE pigs compared to CTRL pigs. Reduced vitamin D consumption followed by reduced intestinal absorption of calcium and phosphorus is the most plausible explanation why CASE pigs had lower serum calcium and

phosphorus than CTRL. Other differential causes for low calcium and phosphorus, such as chronic renal disease and acute pancreatitis were not observed in any pig. Similarly, in the Pittman study, PFTS and fasted pigs had lower serum calcium and phosphorus than the control pigs.¹⁵³ It should be noted that although not significantly different, sodium and chloride were also lower in CASE compared to CTRL pigs in the present study ($0.01 < P < 0.05$ for both, data not shown) and were most likely caused by anorexia as well.

The lower serum glucose levels in CASE pigs were most likely the result of energy deprivation due to anorexia. The Pittman and Buzzard studies also revealed similar findings.^{19,153} However, in a study of mini-pigs, a 24 hour period of fasting did not cause significant changes in serum chemistry including glucose.¹⁹² Thus, consistent with the clinical observation, the CASE pigs had likely experienced prolonged anorexia at the time of sample collection.

Elevated GLDH and AST are indicative of hepatocellular damage. Serum GLDH is mainly derived from hepatocytes, whereas skeletal and myocardial muscle damage can also contribute to increased AST. In the absence of increased CK and no cardiac lesions in CASE pigs, the increased AST is most likely hepatic in origin. However, the predicted mean of AST in CASE pigs was within the normal reference range, which indicates that the hepatocellular damage was mild and unlikely to have caused the clinical signs associated with PFTS. In human patients with anorexia nervosa, acute hepatic damage occurs due to the poor nutritional status.¹⁶³ The same may also apply to PFTS. Unfortunately, the reference range of GLDH in pigs is not available.

No differences in IL-1 β , a proinflammatory cytokine, were detected among groups.

Unfortunately the reagents needed for measuring TNF- α and IL-6, two other proinflammatory cytokines, were not available for the FMIA assay used in this experiment, and insufficient serum

was available to enable testing by other methods. It should be noted that the normal leukogram in this study does not support an overt inflammatory condition in PFTS-CASE pigs. Thus, although an association between PFTS and high proinflammatory cytokines cannot be absolutely ruled out, it is not likely based on the results of this study. CASE pigs from both farm-types had higher serum IL-12 (a Th1 cytokine) than CTRL, which suggests a stimulated cellular immune response in CASE pigs.

6.5. Conclusions

In conclusion, differences in clinical pathology findings of PFTS-CASE compared to other pigs in the current study were most likely caused by prolonged anorexia and dehydration, and no other underlying anatomical pathology was indicated except for mild hepatocellular damage, which could also be the result of anorexia. Additionally, PFTS was not associated with high systemic IL-1 β , a hallmark of a systemic proinflammatory reaction, but was associated with elevated IL-12, one indicator of a stimulated Th1 response.

Table 6.1. Estimated values of serum parameters with significant interaction between farm type and pig type

	PFTS farms (Est. mean±SE)		Partial farms (Est. mean±SE)		P values			Reference ranges
	CASE	CTRL	CASE	CTRL	Farm Type	Pig Type	Interact	
RBC	7.7±0.1 ^a	6.5±0.2 ^b	6.1±0.2 ^{bc}	5.9±0.2 ^c	<0.0001	<0.0001	<0.0001	5.3-8.0 10 ¹² /L
Hgb	136.2±3.6 ^a	109.7±5.0 ^b	103.2±5.8 ^b	102.2±6.0 ^{bc}	0.005	<0.0001	<0.0001	90-140 g/L
Hct	0.44±0.01 ^a	0.36±0.02 ^b	0.35±0.02 ^b	0.35±0.02 ^b	0.025	<0.0001	<0.0001	0.26-0.41 L/L
MCHC	308.0±1.0 ^a	297.4±1.0 ^b	291.4±1.0 ^b	291.9±1.0 ^b	0.145	<0.0001	<0.0001	320-360 g/L
Urea	7.6±1.1 ^a	5.0±1.2 ^b	4.1±1.1 ^b	3.3±1.2 ^b	0.002	<0.0001	0.219	2.9-8.9 mmol/L
Total Bilirubin	9.8±1.2 ^a	1.9±1.3 ^b	3.2±1.2 ^b	2.4±1.2 ^b	<0.0001	0.05	<0.0001	0.9-3.4 g/L
Direct Bilirubin	1.7±1.2 ^a	0.6±1.3 ^b	1.1±1.1 ^c	1.0±1.1 ^b	0.794	<0.0001	<0.0001	0.0-3.4 g/L
Indirect Bilirubin	7.9±1.2 ^a	1.4±1.3 ^b	2.2±1.1 ^b	1.4±1.3 ^{bc}	<0.0001	0.008	<0.0001	0.0-3.4 g/L
Albumin	36.7±1.0 ^a	33.2±0.9 ^b	32.3±2.1 ^b	33.2±1.8 ^b	0.302	<0.0001	<0.0001	19-39 g/L

Est.=estimated; RBC=red blood cell; Hgb=hemoglobin; Hct=hematocrit; MCHC= mean corpuscular hemoglobin concentration

Within row, different letter superscripts represent the results of post hoc pairwise comparison ($P<0.01$)

For case pigs from PFTS farms, control pigs from PFTS farms, case pigs from partial farms and control pigs from partial farms, n=32, 16, 25 and 12 respectively for hematological parameters; n=40, 20, 25 and 12 for all other parameters.

Table 6.2. Estimated values of serum parameters with pig type as the only significant predictor

	Case pigs (n=65)		Control pigs (n=32)		Reference	Units	<i>P</i> values
	Estimated Mean	SE	Estimated Mean	SE			
Calcium	2.5	0.0	2.7	0.1	2.02-3.21	mmol/L	0.002
Phosphorus	2.4	1.0	3.0	1.0	1.46-3.45	mmol/L	<0.0001
Glucose	4.7	0.3	5.8	0.3	3.5-7.4	mmol/L	<0.0001
AST	54.5	1.1	46.2	1.1	21-94	U/L	0.009
Vitamin D	6.5	1.2	14.2	1.1	25-30	mg/L	<0.0001

AST= aspartate transaminase

Table 6.3. Frequency of pigs with detectable serum GLDH (> 2 U/L) and BHB (>0.1mmol/L)

	PFTS-CASE (%)	All other pigs (%)	Fisher's exact <i>P</i>
	n=40	n=57	values
GLDH (>2U/L)	20 (50)	0 (0)	<0.0001
BHB (>0.1mmol/L)	29 (76.3)	4 (7)	<0.0001

GLDH= glutamate dehydrogenase; BHB=beta hydroxybutyrate

In each role, values with different letter suffixes are statistically different by post hoc pairwise comparison ($P<0.01$)

7. Snatch-farrowed, porcine-colostrum-deprived (SF-pCD) pigs as a model for swine infectious disease research

In the works presented in Chapters 4 to 6, the clinical and anatomical pathology of PFTS were characterized, and the relevant pathogens tested were determined to not be the likely etiology of PFTS. Reproducing PFTS in susceptible pigs will be a powerful and unequivocal way of proving it is an infectious disease, even without identification of the causative agent. However, a reliable pig model needs to be first developed prior to inoculation studies. In Chapter 7, efforts were put into developing a snatch-farrowed, porcine-colostrum-deprived pig model in preparation for a PFTS inoculation study.

Chapter 7 has been published and is reproduced here with the permission of the copyright owner (Canadian Veterinary Medical Association).

Huang, Y., D.M. Haines, and J. Harding, Snatch-farrowed, porcine-colostrum-deprived (SF-pCD) pigs as a model for swine infectious disease research. *Can J Vet Res*, 2013. 77(2): p. 81-88.

All authors contributed to the manuscript writing. Huang, with assistance from Harding, performed the experiment and analyzed the data. Haines assisted in the design of the composition of the preweaning liquid formula and quantification of the bovine immunoglobulin concentrations.

7.1. Introduction

In porcine research, especially that investigating infectious diseases, obtaining pigs that are free of porcine pathogens is essential. Currently, three main methods are used to obtain such pigs: conventional pigs tested free for antigen and antibodies of certain specific pathogens (SPF), cesarean-derived colostrum-deprived (CDCD), and the gnotobiotic or germ-free technique. The conventional SPF method chooses pigs that are tested negative for the pathogen of interest. The advantage of this method is its convenience, low requirement for techniques and cost efficiency. However, when the research requires freedom of infection with pathogens that are highly prevalent in pig populations, for instance porcine circovirus type 2 (PCV2), the conventional SPF method may be inadequate, as most pigs have antibodies against these pathogens, either maternal or acquired or are actively infected with the pathogen of interest. As a result, researchers may have to screen a large number of farms and pigs to obtain reliable pig source and select pigs after the waning of maternally-derived antibodies. The CDCD and gnotobiotic methods have been used to successfully obtain pigs for infectious disease studies. Both methods use a Cesarean-section to derive term piglets from pregnant sows. CDCD pigs are raised in sterile compartments for several days and then in a clean room.¹⁸⁹ Gnotobiotic pigs are raised entirely in sterile compartments. Although CDCD and gnotobiotic derivation are reliable methods of obtaining pathogen-free pigs, they have several disadvantages including the need for surgery, specialized facilities, sterile compartments, and are more costly than the SPF method. In addition, the duration of gnotobiotic experiments is limited to only a few weeks, after which the pigs out-grow the sterile compartments. There is also a risk that cesarean-derived pigs, especially if delivered prior to full term, do not fully experience the prenatal serum cortisol surge that occurs in vaginally delivered pigs.¹⁷⁴ The prenatal cortisol surge plays an important role in tissue

maturation, immunoglobulin absorption, surfactant production and the deposition of glycogen in muscles and liver.⁴⁹ This helps to explain why caesarian-derived piglets experience higher morbidity and mortality than in naturally born cohorts. Further, there is debate as to whether gnotobiotic pigs have the same immunological responses as natural-born pigs, because they are not exposed to microbial programming as are piglets reared in natural environments.¹⁸

Recent attempts to raise snatch-farrowed, porcine-colostrum-deprived (SF-pCD) pigs using bovine colostrum provided an alternative method for obtaining susceptible pigs for infective disease experiments.^{12,141} In this method, pigs are fed bovine colostrum for 3 days, a porridge of milk replacer and dry feed from day 4 to 14, and weaned on to dry diet on day 15. The advantages of this method are that the pigs experience natural vaginal delivery and that their intestines are colonized by bacteria, thus the piglets are more representative of conventional pigs compared to CDCD or gnotobiotic pigs. However, survival rates in these studies were at most 80% due mostly to mortality from *E. coli*- or *Staphylococcal*-septicemia.^{12,141} A further disadvantage was that pigs were weaned at 15 days of age, much younger age than in the commercial industry and at an age when there may not be enough digestive enzymes to efficiently cope with solid feed.

In this paper, a method of raising SF-pCD pigs using commercially available bovine colostrum products (HeadSTART™ and Calf's Choice Total™ HiCal, The Saskatoon Colostrum Company Ltd., Saskatoon Canada) is described. This method allows pigs to be weaned at 21 days of age with 100% survival, and produces pigs free of major porcine pathogens and maternal antibodies such that they are fully susceptible to infectious pathogen challenge or receptive to vaccination at a young age. This method is an alternative to Cesarean-derived methods to produce colostrum-deprived pigs for purposes of infectious disease research.

7.2. Material and methods

7.2.1. Snatch-farrowing, animal care and experimental design

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Pregnant sows at the Prairie Swine Center (PSC) Inc., Saskatoon, SK. were used as the source of SF-pCD pigs. Parturition was induced by intramuscular injection of a commercial prostaglandin analogue (Planate, Schering Canada Inc., Pointe-Claire, Quebec, Canada) on day 115 of pregnancy. The perivulvar area of the sows and the pens in which the sows were housed were washed twice on the day before expected parturition with warm water and sprayed with an iodine disinfectant (Prepodyne, West Penetone Inc., Ville d'Anjou, Quebec, Canada). Before parturition, a clean drape was placed behind the sow to reduce the risk of environmental contamination of the piglets. During parturition, piglets were snatched before contacting the floor, farrowing equipment or barn facilities. The umbilical cords were clamped and disinfected with Prepodyne. Piglets were, within 1 minute of birth, placed into sealed plastic containers fitted with a High-Efficiency Particulate Air (HEPA) filter allowing the provision of filtered fresh air while transported to a biosecurity level 2 animal care room with positive pressure ventilation at the University of Saskatchewan. The animal room was disinfected twice at 24 hour intervals before pig entry: first with 7% hydrogen peroxide solution (Peroxigard, Bayer Inc., Toronto, Ontario, Canada) and followed with 1% Virkon solution (Antec International Limited., Lavaltrie, Quebec, Canada). Piglets were raised in groups of 2 (experiment 2) or 3 (experiment 1) per pen on elevated plastic floor. The concrete floor beneath the plastic flooring was washed 2 to 3 times each week in experiment 1, and daily in experiment 2. The room temperature was set at 30⁰C

from day 1 to 21, and decreased by 1⁰C weekly thereafter. Before weaning, a heat lamp was provided for each pen and the height of the lamps was adjusted according to the pigs' comfort level (i.e. pigs sleep comfortably on their sides under the heat lamp exposing their abdomen).

After entry, piglets were fed a liquid diet (Table 7.1) hourly for the first 6 hours, every 2 hours from 6 to 24 hours of age, and 4 times per day (8:00 am, 12:00 am, 4:00 pm and 10:00 pm) thereafter until weaning. The pigs were initially bottled-fed, while the liquid diet was also provided in a liquid feeder (Miller Manufacturing Company, Eagan, MN; product number: BPW4) in the pen to encourage drinking from the feeder. Once a pig was observed to be drinking from the feeder, bottle-feeding was discontinued for that pig. All pigs began to drink from the feeder within 48 hours of age. The liquid diets contained combinations of the following ingredients: spray-dried bovine colostrum powder containing at least 25% bovine IgG (HeadSTARTTM, The Saskatoon Colostrum Company, Saskatoon, SK, Canada), spray-dried bovine colostrum powder containing at least 14% bovine IgG (Calf's Choice TotalTM HiCal, Saskatoon Colostrum Company, Saskatoon, SK, Canada), commercial pig milk replacer (WetNurse, Prairie Micro-Tech Inc, Regina, SK, Canada), K88 E. coli hyperimmunized egg yolk protein (Hyper-egg; J. H. Hare & Associates Ltd, Winnipeg, MB, Canada) and an oral iron supplement (Enfamil® Fer-In-Sol® syrup, Mead Johnson & Company, Canada; 30 mg elemental iron per 5 ml). The targeted dry matter fed to each pig in the liquid diet gradually increased from 66 g/day on day 1 to 500 g/day on day 20, and the feeding volume increased from 150 ml/d to 3300 ml/d. The exact feeding amount and volume varied somewhat based on appetite. Pigs were weaned on day 24 (experiment 1) or day 21 (experiment 2) to a custom starter diet absent of porcine by-products (Table 7.2), with the exception of bovine colostrum fed to the treatment group in experiment 2.

No prophylactic antibiotic treatment was employed before the pigs enter the animal rooms, nor before weaning. No antibiotics were supplemented in the starter feeds used in these experiments. In experiment 1, when pigs developed fever higher than 40°C, oxytetracycline (Bio-mycin, Boehringer Ingelheim, Burlington, ON, Canada) was given intramuscularly (1ml, 20 mg/ml) to release the clinical symptoms of septicemia.

Experiment 1 aimed to compare the effects of two different liquid diet formulations on the health of SF-pCD pigs. The 12 SF-pCD pigs used in this experiment were conveniently placed (3 per pen) in the order they arrived at the facility into 4 feet x 6 feet (1.23m x 1.85m) pens equipped with one liquid feeder. Unlimited access to water was provided by a nipple drinker. All pigs were housed in the same room throughout the experiment. Two systematically selected pens (pens 1 and 2; 3 pigs per pen) (RPL; n=6 pigs) were fed a liquid diet comprised mainly of bovine colostrum for the first 10 days, then the colostrum was gradually replaced with milk replacer until leaving 5% (w/v) bovine colostrum in the diet (Table 7.1). The remaining 2 pens (pens 3 and 4; 3 pigs per pen) (COL; n=6 pigs) were fed a liquid diet comprised mainly of bovine colostrum throughout days 1 to 20 (Table 7.1). Starter diet was introduced to all the pigs on day 20 and the pigs were fully weaned on day 24. Blood samples were drawn into EDTA tubes from the cranial vena cava 24 hours after the initial bovine colostrum feeding and weekly thereafter from half of the pigs of each group. Rectal temperatures were measured daily from entry to day 33. The experiment was terminated on day 35 when all the surviving animals were euthanized with intravenous barbiturate and necropsied.

Experiment 2 aimed to evaluate the potential benefits of adding bovine colostrum powder to the dry starter diet fed after weaning. Twelve SF-pCD pigs were used in this experiment. Two conveniently selected pigs were placed in each pen. All pigs were kept in the same animal room

throughout the experiment. Before weaning on day 21, all pigs were fed a liquid diet comprised mainly of bovine colostrum (Table 7.1). At weaning, the pens were systematically allocated into 2 groups. Three pens (pens 2, 4 and 6; 2 pigs per pen) (STARTER-COL; n=6 pigs) were fed a dry starter diet devoid of all animal byproducts except 20% (w/w) colostrum (Calf's Choice Total HiCalTM, The Saskatoon Colostrum Co. Ltd., Saskatoon SK, Canada). The remaining three pens (pens 1, 3 and 5; 2 pigs per pen) (STARTER-CTRL; n=6) were fed the same starter diet as was used in experiment 1 (Table 7.2) without additional colostrum. Blood samples were drawn from all pigs into EDTA tubes from the cranial vena cava 24 hours after the initial bovine colostrum feeding and weekly thereafter. Rectal temperatures were measured daily until day 30, and twice weekly thereafter. Half of the pigs from each group were euthanized by intravenous barbiturate on day 42 and the remaining on day 49. Necropsy examination was performed immediately after euthanasia.

7.2.2. Packed cell volume and plasma total protein

In both experiments, packed cell volume (PCV) was determined in the whole blood samples by the capillary tube method and the plasma total protein concentration was determined by refractometry.

7.2.3. Histopathological examination of tissues

Internal organs were collected at necropsy, preserved in 10% formalin and processed for histopathological examination. Tissues examined included brain, nasal turbinate, salivary gland, tonsil, thymus, lung, heart, bronchial lymph node, stomach (fundus and pylorus), duodenum, jejunum, ileum, spiral colon, cecum, mesenteric lymph node, adrenal gland, kidney and superficial inguinal lymph node.

7.2.4. Adjunct tests for porcine pathogens

In both experiments, the presence of pathogen-specific antibodies was examined in plasma collected immediately prior to necropsy by ELISA using commercial kits employed according to the manufacturer's instructions by Prairie Diagnostic Services Inc. (PDS, Saskatoon, Canada). The samples were tested for antibodies to swine influenza virus (SIV; Swine Influenza Virus Antibody Test Kit H1N1 [Part number: 99-06731] and Swine Influenza Virus Antibody Test Kit H3N2 [Part number: 99-09332], IDEXX Laboratories, Inc., Westbrook, Maine, USA) and porcine reproductive and respiratory syndrome virus (PRRSV; PPRS X3 HerdChek, Porcine Reproductive and Respiratory Virus Antibody Test Kit [Part number: 99-18070], IDEXX Laboratories, Inc., Westbrook, Maine, USA). Porcine parvovirus (PPV) antibody was tested by PDS Inc. using an in-house haemagglutinating inhibition assay adapted from a previously described protocol.²⁰ Briefly, porcine parvovirus propagated in the laboratory from a diagnostic isolate was used for the haemagglutinating antigen, which was incubated at a concentration of 6 HA units with two fold dilutions of serum starting with an initial dilution of 1:20. Haemagglutinating activity was assessed using chicken red blood cells. An antibody test for porcine circovirus type 2 (PCV2) by immunoperoxidase monolayer assay (IPMA) was performed using a previously described in-house assay.¹¹⁹ In both experiments, the presence of PCV2 DNA in plasma collected at all time points, and in spleen, superficial inguinal lymph node, and tonsil collected at termination was tested by the polymerase chain reaction (PCR).¹¹⁶ Additionally in experiment 2, the presence of DNA of Torque Teno virus (TTV) genogroups 1 and 2 in bone marrow was tested by PCR.⁹² For pigs that died or were euthanized for humane reasons before the termination of the experiment, aerobic and anaerobic bacterial cultures were performed on lung, spleen, and joint fluid or abdominal fluid.

7.2.5. Plasma bovine and porcine immunoglobulin (IgG) concentration

The concentration of bovine (b)IgG in plasma was determined by radial immunodiffusion (RID) as previous described.²⁶ The plasma half life of bIgG was calculated using the following formula, $T_{1/2}=(T*\log2)/\log \frac{ConB}{ConE}$, where $T_{1/2}$ is the half live, T is the total time period, ConB is the beginning bIgG concentration and ConE the ending bIgG concentration. All plasma collected in experiment 2 was also tested for porcine (p)IgG by RID as previous described.¹⁴³ The plasma pIgG concentrations between groups were not compared by statistic methods because the half of the pigs in each group were euthanized on d42 and the remaining on d49, which substantially reduced the statistical power (n=3).

7.2.6. Statistical analysis

Differences in frequencies of lesions between groups were compared by Fisher's exact test. The group difference of plasma bIgG concentration on day 1 was compared by Mann-Whitney U test. The fever-days, defined as total number of days that individual pigs had body temperature greater than (including) 39.5⁰C, were compared between groups by generalized linear models using a Poisson regression using Predictive Analytics SoftWare (PASW) Statistics 18. Probabilities of less than 0.05 were considered statistically significant.

7.3. Results

7.3.1. Experiment 1

All pigs learned to drink from the liquid feeder within 48 hours of entry. Four of six RPL pigs and all COL pigs survived until the termination of the experiment (day 35). One RPL pig was observed to have atresia ani on day 2 and defecated by way of a rectovaginal fistula. On day 16,

it developed lethargy, anorexia, fever (40.4⁰C) and was euthanized by intravenous barbiturate. Acute fibrinous polyserositis and renal petechiation were noted grossly. Histopathological changes were consistent with acute septicemia and *E. coli* was cultured from lung, spleen and joint fluid. A second pig developed lethargy, anorexia and fever (41.4⁰C) on day 22. Gross and histological lesions were indicative of acute septicemia and *E. coli* was also cultured from lung, spleen and abdominal fluid. The *E. coli* isolates from both pigs were not further characterized.

The RPL pigs had significantly more fever-days than COL pigs from day 11 to day 20 (16 fever-days / 50 total pig-days in RPL versus 5/54 in COL, $P = 0.009$) but not from day 1 to 10 or from day 21 to 33 ($P > 0.05$)(Figure 7.1). Two of 4 RPL pigs and 4 of 6 COL pigs had mild neutrophilic infiltration in the mesenteric lymph nodes on histology, but the frequencies were not statistically different ($P > 0.05$). No other pathological changes were observed pigs that survived until the termination on day 35.

RPL pigs had marginally but significantly higher levels bovine IgG in plasma at day 1 and 7 ($P = 0.0495$) even though both groups were on the same diet during this time. Plasma concentrations of bovine IgG were highest 24 h after initial colostrum intake, and decayed rapidly within the first two weeks of life to insignificant levels (Figure 7.2). The half-life of bovine IgG in experiment 1 was 9.2 days. PCV and plasma total protein concentrations remained within normal range throughout experiment 1 (data not shown).

At termination, all the pigs were negative for antibodies to SIV (H1N1 and H3N2), PRRSV and PPV. However, one COL pig was positive for PCV2 antibody at termination. PCV2 DNA was detected in the plasma of this pig from day 21 to day 35, and in spleen and superficial inguinal lymph node collected at necropsy. PCV2 DNA was also detected by PCR in the spleen and

superficial inguinal lymph node collected from one other COL pig, reared in the same pen, although this pig remained negative for PCV2 antibody.

7.3.2. Experiment 2

All pigs learned to drink from the liquid feeder by 48 hours. All pigs in both groups survived until the end of the experiment. The feces of all STARTER-CTRL pigs were of normal consistency throughout the experiment. Four of six STARTER-COL pigs developed semi-liquid diarrhea beginning day 27 (6 days post weaning) which persisted until the end of the experiment. The diarrheic pigs, however, remained alert and healthy otherwise. On necropsy, these pigs had dilated colons and caeca that contained unformed feces. The large intestinal mucosa was reddened and mesocolonic vessels were congested. Histological examination of the colon and cecum of these pigs revealed typhlocolitis with combinations of the following changes: bacterial attachment on the mucosa, degeneration and necrosis of the epithelial cells, congestion, edema and mixed inflammatory cell infiltration of the lamina propria. No adjunct tests were carried out to characterize the etiology of the typhlocolitis. In addition, the pancreases of all STARTER-COL pigs were firm and had a nodular appearance on gross examination. Pancreatic glandular epithelial degeneration and necrosis with regeneration and fibrosis was evident microscopically. The pancreases of the STARTER-CTRL pigs were normal macro- and microscopically, except in one STARTER-CTRL pig that had mild microscopic pancreatic degeneration. Both groups had only 2 fever-days in total.

Similar to experiment 1, plasma concentrations of bovine IgG were highest 1 day after initial colostrum intake, then decreased rapidly within the first two weeks to insignificant levels (Figure 2). The half-life of bovine IgG in experiment 2 was 5.5 days. Porcine IgG was not detectable in

the plasma until day 21 and gradually increased to 5 g/L on day 49 (Figure 7.2). PCV and plasma total protein concentrations of all pigs remained within normal range at all time points (data not shown).

All pigs were negative at termination for antibodies to SIV (H1N1 and H3N2), PRRSV, PPV and PCV2. The absence of PCV2 infection was confirmed by PCR test in terminal plasma and tonsils. TTV1 DNA was detected by PCR in 1/6 STARTER-COL pig and 4/6 STARTER-CTRL pigs. TTV2 DNA was detected by PCR in 1 STARTER-CTRL pig that was also positive for TTV1.

7.4. Discussion

In the current experiments, neonatal SF-pCD pigs were successfully raised on a bovine colostrum-based liquid diet, achieving a survival rate of 100% while remaining serologically negative for PRRS and SIV. Moreover, all but 1 pig remained serologically negative for PCV2 and only 2 pigs had detectable PCV2 DNA detected in serum or tissues in the first of the two experiments.

The bovine colostrum fed in the liquid diet of SF-pCD pigs is likely the main factor contributing to the high survival rate in the current studies. It is well known that the epitheliochorial placentation in pigs and other farm mammals prevent macromolecule transportation from dam to fetuses. Thus, pigs are born hypogammaglobulinemic (e.g. only trace amounts of immunoglobulin in the blood). As a result, it has been shown that neonatal pigs have a low survival rate when neither sow colostrum nor immunological supplements derived from other sources (e.g. bovine colostrum) were fed, in spite of intensive treatment with antibiotics.⁵⁸ In other studies employing bovine colostrum in neonatal pigs, colostrum was fed for only several days,^{12,58,141} and the survival rates of the pigs were at most 80%.^{12,58} In the present experiments,

the use of commercially available spray-dried bovine colostrum powder allows more protracted use of the colostrum and enables this SF-pCD technique to be easily adopted by other laboratories. It is clear from our results that providing bovine colostrum to pigs after gut closure provided health benefits up to weaning at 3 weeks of age. Although immunoglobulins cannot be absorbed in significant quantity after gut closure and the blood half lives of bovine IgG in these experiments are shorter than that of porcine IgG (at least 12 – 14 days),¹⁶⁶ they and the other antimicrobial substances present in colostrum may provide local immunity in the gastrointestinal tract (GIT).

It is noteworthy however, that bovine colostrum fed after weaning to SF-pCD pigs was associated with persistent diarrhea, typhlocolitis and pancreatic degeneration in experiment 2. The mechanism behind this phenomenon was not determined in this experiment. The level of fat in the diet supplemented with the colostrum was 8.4 % compared with 1.5 % in the diet without the supplementation (Table 7.2). The additional colostrum powder in the diet after weaning without consideration of the fat levels in the diet may exceed the capacity of the pancreatic enzymes and caused maldigestion and diarrhea (likely osmotic). Irrespective of the reason, the finding that there was no benefit to supplementation of SF-pCD pigs after weaning with bovine colostrum substantially reduces the cost of raising these pigs.

The normal PCV of the SF-pCD pigs indicate that oral iron supplementation was sufficient to prevent anemia. The industry-standard practice of parenteral iron dextran administration was not used in this experiment, since there was a concern that any injection to these colostrum-deprived pigs would increase the risk of septicemia. For the same reason, only half of the pigs were bled in experiment 1. However, in experiment 2, when all the pigs were bled weekly, no septicemia occurred in any pigs. Thus, injections and bleeding can be performed in SP-pCD pigs without

any health compromise, provided these activities are performed hygienically. Indeed, in a subsequent SF-pCD experiment that our laboratory has undertaken iron dextran administration was performed intramuscularly in the neck without any ill effects (data not shown).

The roles of IgY-K88, which reportedly prevented K88 (F4)-*E. coli* diarrhea,¹⁰⁹ were not characterized in this experiment. The inclusion of this component in the diet was to help avoid potential illness due to K88-*E. coli*. F4 colibacillosis is a common disease of young pigs in the commercial swine industry, and vaccination of dams prior to farrowing is routinely performed.

It is noteworthy that in experiment 2, pyrexia was rare, in contrast to experiment 1. This may be due to a higher level of sanitation during experiment 2 as the concrete under the plastic floor was cleaned more frequently (daily) than in experiment 1 (2 to 3 times weekly), discouraging the accumulation of fecal micro-organisms in the environment. The benefit of high sanitation is further emphasized by the fact that when SF-pCD pigs were raised on a concrete floor bedded with straw 4/9 pigs developed *E. coli*-diarrhea and -septicemia.¹⁴¹ Thus, good hygiene practices are strongly recommended for the health of SF-pCD pigs.

The results of this experiment demonstrate that it is possible to raise SF-pCD pigs that remain free of porcine pathogens including PRRS, SIV, PPV and PCV2. The source farm in this experiment was serologically free of PRRS virus, but positive for PCV2, SIV and PPV.

Snatch-farrowing, preventing contact with barn facilities, minimizing exposure to barn air, and disinfection of the sows and piglets are likely all critical in preventing horizontal transmission of these pathogens to SF-pCD pigs. While the source of PCV2 infection in two experiment 1 pigs was not definitively identified, a retrospective evaluation of our biosecurity protocols guiding entry into the animal care room and the timing of infection suggest the introduction of a bottle of

antibiotic that had been previously used in a PCV2 positive farm could be implicated. It should be noted that, in experiment 1, all the pigs remained PCV2 negative before day 21, which indicates that vertical transmission of PCV2 was unlikely. All pigs remained PCV2 negative in experiment 2 after biosecurity measures were improved. Thus, it is possible, although not guaranteed, to obtain PCV2-negative pigs from a PCV2-positive farm using the SF-pCD method provided piglets are not infected prenatally. A recent study showed that 39.9% of the piglets in 5 North America farms were born PCV2-viremic.¹⁸² Although most of the piglets were born from gilts in that experiment, which may be more likely to vertically infect their progeny *in utero*, the putatively high rate of viremia at birth on some farms highlights that PCV2-free status is not guaranteed. The authors recommend excluding PCV2-viremic sows to increase the probability of obtaining PCV2-free piglets.

Similarly, the presence of TTV1 and TTV2 in some SF-pCD pigs was not unexpected since vertical transmission of TTV had been reported.^{113,159} In this regard, even gnotobiotic derivation cannot guarantee freedom of TTV infection, or that of other vertically transmitted pathogens.

The SF-pCD method has advantages over the conventional SPF method in obtaining pathogen-free pigs, and is less technically demanding than CDCD and gnotobiotic models. Since conventional SPF pigs have suckled their dams and remain on farm prior to their inclusion in an experiment, there is a high risk of exposure and possibly infection with organisms circulating on the farm. Moreover, the presence of maternally-derived antibodies against endemic pathogens means SPF piglets cannot be used for experiments until after the decay of passive immunity. Unlike both CDCD and gnotobiotic derivation, SF-pCD derivation requires neither surgery nor sterile compartments. Thus, the technical requirements of this model are low and easily adapted to any laboratory. The natural birth of SF-pCD pigs allows them to be

exposed to the vaginal flora, which may cause exposure and potential infection by the vaginal microbiota and pathogens if present. The technique is also animal welfare friendly, without need to sacrifice donor sows post-surgery.

In conclusion, this experiment established a method to raise SF-pCD pigs with high health and survival that balances convenience, freedom of major pathogens, animal welfare, cost and pathogen susceptibility. The SF-pCD model is a good alternative to current methods for producing pigs for infectious disease research. The main disadvantages of raising SF-pCD pigs are the intensive labour associated with bottle feeding piglets during the first 48 hours, the risk of contamination by vaginal microbiota and the risk of septicemia if the environment is not hygienic.

Table 7.1. Ingredients in the liquid diets fed until weaning to snatch-farrowed, porcine-colostrum-deprived pigs in experiments 1 and 2

Ingredient ^a	Experiment 1								Experiment 2	
	Days 1 to 3		Days 4 to 10		Days 11 to 15		Days 16 to 23		Days 1 to 3	Days 4 to 21
	RPL	COL	RPL	COL	RPL	COL	RPL	COL		
Colostrum A (%, w/v)	20	20	0	0	0	0	0	0	20	0
Colostrum B (%, w/v)	0	0	15	15	Reduce to 5	15	5	15	0	15
Milk replacer (%, w/v)	0	0	0	0	Increase to 13	0	13	0	0	0
Iron (mg/kg milk solids)	250	250	250	250	250	250	250	250	250	250
IgY K88 (g/pig/d)	2	2	2	2	2	2	2	2	2	2
Warm water (L/pig/d)	0.3–0.53		0.67–1.67		1.84–2.5		2.67–3.33		0.3–0.53	0.67–3.33

^a Colostrum A — HeadSTART, The Saskatoon Colostrum Company, Saskatoon, Saskatchewan; Colostrum B — Calf's Choice Total HiCal, The Saskatoon Colostrum Company; milk replacer — WetNurse, Prairie Micro-Tech, Regina, Saskatchewan; iron — Enfamil Fer-In-Sol syrup (30 mg of elemental iron per 5 mL), Mead Johnson & Company, Ottawa, Ontario; IgY K88 — Hyper-Egg K88; J.H. Hare & Associates, Winnipeg, Manitoba.

RPL — Replacement: dietary colostrum was gradually replaced with milk replacer from day 11 until 5% colostrum remained in the diet; COL — Colostrum: the diet consisted mainly of bovine colostrum until day 20.

Table 7.2. Ingredients and nutrient levels in the dry starter diet fed to the pigs after weaning

Ingredients	Experiment 1	Experiment 2	
	(%)	STARTER-COL (%)	STARTER-CTRL (%)
Wheat	30.90	44.80	30.90
Colostrum B	0	20.00	0
White fish meal	8.61	0	8.61
Oat groat	15.00	15.00	15.00
Whey permeate	20.00	11.40	20.00
Soybean meal	15.00	3.60	15.00
NuPro ^a	5.00	0	5.00
Canola oil	3.00	2.00	3.00
Limestone	0.43	0.90	0.43
Salt	0.44	0.63	0.44
Monocalcium phosphate	0	0.63	0
Zinc oxide	0.40	0.40	0.40
Lysine	0.47	0.36	0.47
Starter microbial phytase	0.20	0.20	0.20
Choline chloride	0.08	0.08	0.08
Methionine	0.25	0.03	0.25
Threonine	0.20	0	0.20
L-tryptophan	0.03	0	0.03
Nutrients^b			
Crude protein	21.7	21.5	21.7
Crude fat	1.5	8.4	1.5
Crude fibre	5.6	1.6	5.6
Digestible energy (Mcal/kg)	3.69	3.76	3.69
Net energy (Mcal/kg)	2.62	2.76	2.62

Ingredients	Experiment 1	Experiment 2	
	(%)	STARTER-COL (%)	STARTER-CTRL (%)
Calcium	0.9	0.9	0.9
Available phosphorus	0.8	0.7	0.8
Sodium	0.47	0.45	0.47
Total lysine	1.58	1.64	1.58
Total threonine:lysine	0.64	0.77	0.64
Total methionine+cystine:lysine	0.58	0.61	0.58
Total tryptophan:lysine	0.17	0.20	0.17

^a Alltech Canada, Guelph, ON

^b As-fed basis; estimated 90% dry matter.

STARTER-COL — Diet devoid of all animal by-products except 20% (w/v) colostrum B;

STARTER-CTRL — Same starter diet as used in experiment 1.

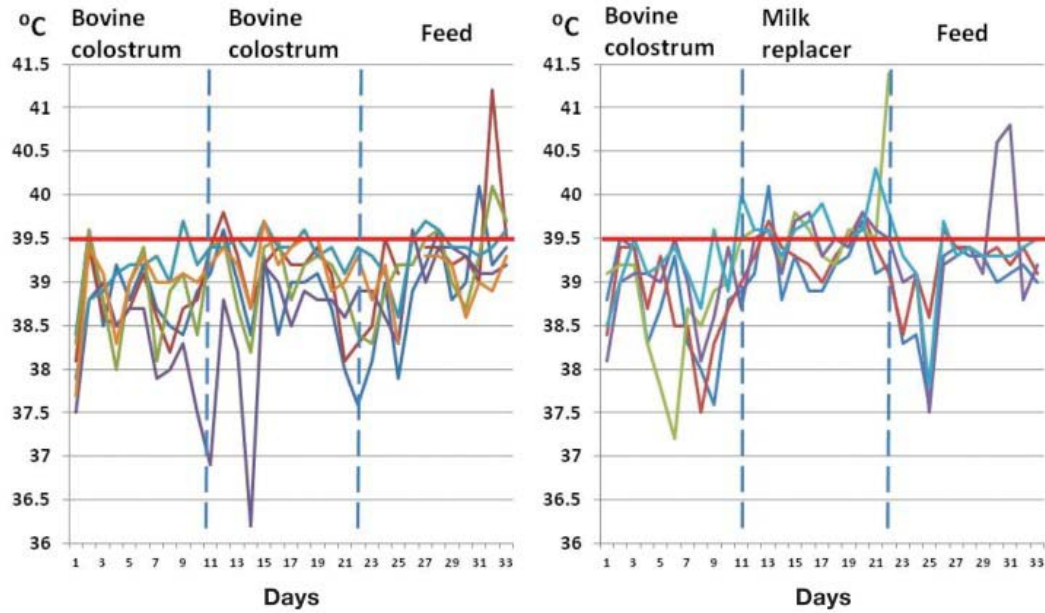


Figure 7.1. Body temperatures of snatch-farrowed, porcine-colostrum-deprived (SF-pCD) pigs in experiment 1. Between days 11 and 20 of life the pigs fed bovine colostrum as the main component of their diet (left panel) had significantly fewer days of fever ($P = 0.009$) than the pigs for which the colostrum was gradually replaced with milk replacer from day 11 (right panel) before weaning to a dry feed.

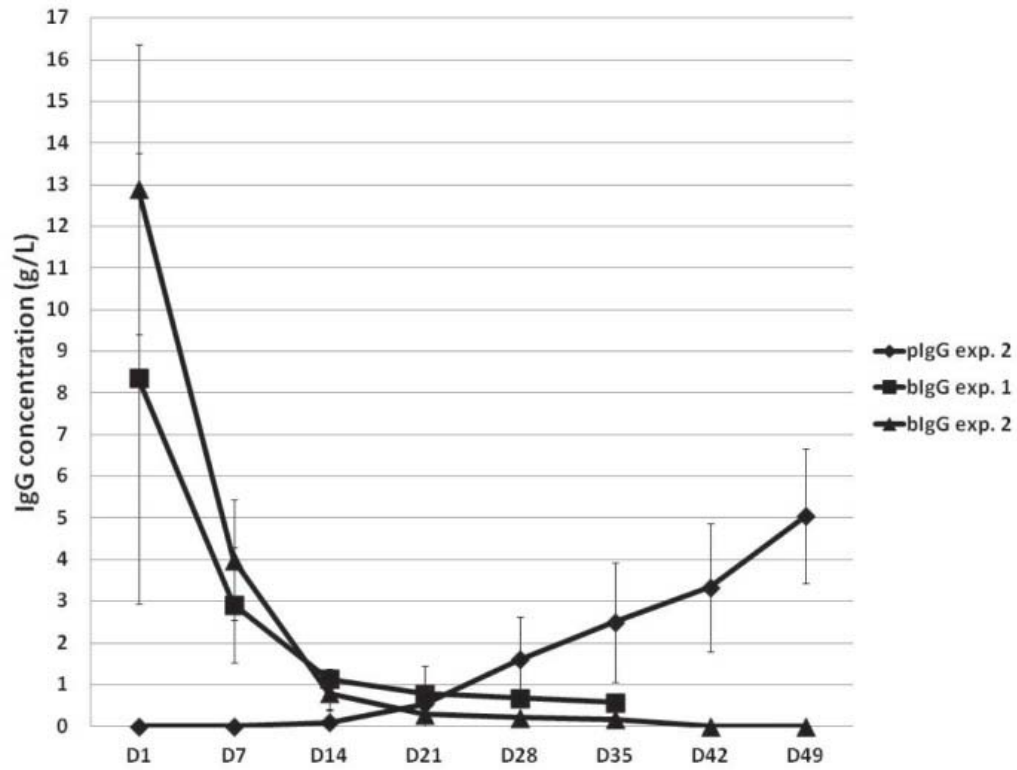


Figure 7.2. Plasma concentrations of bovine and porcine immunoglobulin G (bIgG and pIgG) in the SF-pCD pigs in experiments 1 and 2. Squares indicate mean values for experiment 1. Triangles indicate mean values for bIgG and diamonds mean values for pIgG in experiment 2. The vertical lines represent standard deviations.

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8. Snatch-farrowed porcine-colostrum-deprived (SF-pCD) pigs possess similar cellular and humoral immune responses to *Mycoplasma hyopneumoniae* vaccination compared to their farm-raised siblings

Chapter 7 successfully established a reliable protocol to raise SF-pCD pigs. Since these pigs are porcine-colostrum-deprived, they are free of antibodies to porcine pathogens, and thus, should be susceptible to inoculation with these agents. If it can be further shown that SF-pCD pigs grow and have the same level of immunity as siblings raised on farm, it will support that SF-pCD pigs are suitable for use as in pathogen inoculation studies. The experiments in Chapter 8 were designed to address this rationale.

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Huang, Y., D.M. Haines, and J. Harding. Snatch-farrowed porcine-colostrum-deprived (SF-pCD) pigs possess similar cellular and humoral immune responses to *Mycoplasma hyopneumoniae* vaccination compared to their farm-raised siblings. Journal to be determined, 2013. Manuscript in preparation.

8.1. Introduction

A reliable animal model is critical to the success of research that attempts to reproduce infectious disease in experimental settings. In pigs, there are currently three types of models frequently used in such studies: the conventional specific pathogen free (SPF) model, the cesarean-derived colostrum-deprived (CDCD) model, and the gnotobiotic model. However, conventional SPF pigs are not suitable for studies of diseases caused by highly prevalent pathogens, since one needs to test large numbers of pigs to identify adequate numbers of SPF pigs, and the presence of maternal antibodies precludes challenge of young pigs with pathogens. Both CDCD and gnotobiotic models require surgery to deliver piglets and sterile compartments to raise them. In the case of gnotobiotic pigs, they are raised entirely in sterile compartments. Thus, the duration of experiments is limited because pigs may outgrow the space of the compartments. It was in this context that we optimized a previously published snatch-farrowed porcine colostrum-deprived (SF-pCD) pig model^{12,141} to achieve 100% survival and provided an alternative model for infectious disease research in pigs.⁷⁷ SF-pCD pigs were raised on a bovine-colostrum-based liquid diet before weaning, and a post-weaning diet that was free of porcine byproduct.⁷⁷

Knowledge about the immunity of pigs raised for experimental use is limited. It has been demonstrated that gnotobiotic pigs not colonized by any bacteria failed to produce serum IgG and IgM to T cell dependent and type-2 T cell independent antigens, but those colonized even by one single strain of *Escherichia coli* did.¹⁸ This showed that gnotobiotic pigs differ from conventional pigs immunologically. This raises questions regarding the extent to which knowledge generated through the use of gnotobiotic pigs is applicable to field situations. Similarly, it is also unknown whether SF-pCD pigs are representative of conventional, farm-raised pigs in terms of their immunological responses. The objective of the current

experiment was to compare the growth performance, and cellular and humeral immune responses to *Mycoplasma hyopneumoniae* (Mhyo) vaccination between SF-pCD pigs and their farm-raised siblings.

8.2. Methods

8.2.1. Animal procedures

Twenty five neonatal pigs were hygienically snatch-farrowed from four sows at the Prairie Swine Center (PSC) Inc. as previously described.⁷⁷ PSC Inc. is historically negative for Mhyo and pigs were not vaccinated against this agent. As soon as all pigs were collected, twelve pigs (SF-pCD) were transferred to the Animal Care Unit (ACU) at the Western College of Veterinary Medicine (WCVN), University of Saskatchewan and raised according to a previously developed protocol in which commercial bovine colostrum was used as the main diet before weaning.⁷⁷ Thirteen siblings, blocked by dam, sex and subjective birth sizes remained on-farm to be raised by their biological sows (FARM). Piglets not involved in this study were fostered on to the experimental dams as required so that the litter sizes were 10-12 at farrowing. On day (D)2, 200 mg parenteral iron dextran (Ferroforte, Bimeda-MTC Animal Health Inc., Cambridge ON) were given to each pig. On D20, all pigs were weaned and fed a dry starter diet free of porcine byproducts until termination of the study on D44. The SF-pCD pigs were raised in groups of two throughout the study in 1.23 m x 1.85 m pens equipped with one liquid feeder per pen. The FARM pigs were weaned into two 2.5 m x 1.04 m nursery pens in groups of 6 and 7. All pigs were weighed on D1 (day of birth was recorded as D0) and then twice weekly before weaning, and once weekly after weaning.

On D7, pigs were injected intramuscularly with 2 ml of Mhyo bacterin (RespiSure, Zoetis

Animal Health, Inc. Kirkland, QC, Canada). On D26, a booster vaccination was administered in the same manner. Blood samples were taken from the cranial vena cava on D1 and then weekly after (D7, 13, 20, 26, 33 and 40). The main events in this experiment are shown in Figure 8.1.

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

8.2.2. IFN- γ ELISPOT assay

Peripheral blood mononuclear cells (PBMC) were isolated from D26 and D40 blood samples. Whole blood was collected in a sodium-heparin blood collection tube, diluted 1:1 with PBS (pH=7.4) and was overlaid on to Ficoll-paque Plus (GE Healthcare Bio-Sciences Corp. Piscataway, NJ, USA). PBMC were then separated by differential centrifugation at $400 \times g$ at 20°C for 30 minutes. After hypotonic lysis of red blood cells, the PBMC were washed once in PBS and once in culture medium (Gibco® RPMI Media 1640, Life Technologies Inc., Burlington, ON, Canada) with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin. Cells were stained with Trypan blue and counted on a hemocytometer. MultiScreen_{HTS} filter plates (EMD Millipore Corporation, Billerica, MA, USA) were coated with $10 \mu\text{g/ml}$ mouse anti-porcine INF- γ monoclonal antibodies (Mabtech Inc. Mariemont, OH, USA) and incubated overnight at 4°C . Subsequently, plates were washed 3 times with PBS and blocked with RPMI media containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C for 1 hour. PBMC (8×10^5) cells were then dispensed into each well and incubated with $10 \mu\text{g/ml}$ Mhyo antigen (courtesy of M. Raymond, Iowa State University), $5 \mu\text{g/ml}$ ConA or media alone for 40 hours at 37°C in the presence of 5% CO_2 . Each sample was assayed in triplicate. Cells were removed and the plates washed twice with distilled water and 3 times with PBS with 0.01%

Tween 20 (PBST) before being incubated with 1 µg/ml biotinylated mouse anti-bovine IFN-γ mAb (Mabtech Inc. Mariemont, OH, USA) for 1 hour at room temperature. The plates were washed 4 times with PBST and incubated with 0.5 ng/ml streptavidin alkaline solution (Jackson ImmunoResearch Laboratories Inc. West Grove, PA, USA) in PBST with 1% bovine serum albumin (BSA) at room temperature for 45 minutes. The plates were then washed 3 times with PBST and twice with PBS. Spots were developed by adding SIGMAFAST™ BCIP®/NBT (Sigma-Aldrich Corporation, St. Louis, MO, USA) to each well according to the manufacturer's instructions. The number of spots each in each well were counted by AID Elispot Reader ELRIFL07 (Autoimmun Diagnostika GMBH, Strasbourg, Germany). Numbers of Mhyo-stimulated IFN- γ-secreting cells were calculated by subtracting the numbers of spots in media wells from those in Mhyo wells and expressed as numbers of IFN- γ-secreting cells per million PBMC.

8.2.3. Serum Mhyo antibody ELISA

Serum Mhyo antibody titers were determined using the IDEXX Mhyo Ab ELISA Test kit (IDEXX Laboratories, Inc., ME, USA) according to manufacturer's instructions. Testing was conducted at Biovet Inc. (Saint-Hyacinthe, QC, Canada). Briefly, a strong positive swine control serum was serially diluted in phosphate buffered saline (PBS) from 1:40 (working dilution) to 1:5,120 and tested using the IDEXX Mhyo Ab ELISA. By convention it was attributed a titer of 2,560 corresponding to its final ELISA positive dilution (sample to positive ratio (S/P) = 0.4). The serum samples were tested at a dilution 1:40 along with the positive control serum dilutions. The S/P ratios of the positive control serum dilutions were plotted against their theoretical antibody titers using curve-fitting software for ELISA analysis (MasterPlex® ReaderFit, Hitachi Solutions America Ltd, CA, USA). The S/P ratios of the samples were plotted the same way and

their titers were automatically generated by the MasterPlex® ReaderFit software.

8.2.4. Serum porcine and bovine IgG concentration

Serum bovine IgG concentrations were determined by radial immunodiffusion (RID) as previously described.⁷⁷ The porcine IgG RID assay was adopted from the bovine assay with a few changes: the antibody used was 3.0% goat anti-swine IgG (H+L) antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), and the standard was purified swine IgG (Bethyl Laboratories Inc., Montgomery, TX, USA). Half life of bovine IgG in SF-pCD pigs was calculated as described previously.⁷⁷

8.2.5. Statistical analyses

Body weights (D1, D20 and D40), Average daily gain (ADG) (overall, pre- and post-weaning), numbers of PBMC secreting IFN- γ (D26 and D40), serum Mhyo IgG levels (D40) and serum porcine IgG concentration (D40) were compared between groups using Generalized Estimation Equations (GEE) accounting for clustering of litters. All models used an exchangeable correlation matrix with a robust variance estimator. Normality and homogeneity of the residues were evaluated. All the analyses were performed by IBM SPSS Statistics version 21. *P* value less than 0.05 were considered statistically significant.

8.3. Results

Results of analysis of weight and ADG are summarized in Table 8.1. Body weights of SF-pCD and FARM pigs did not differ on D1 or at weaning on D20 ($P>0.05$). Similarly, pre-weaning ADG did not differ between groups ($P>0.05$). However, after weaning SF-pCD pigs exhibited superior ADG ($P<0.0001$) and their final body weight was significantly higher than FARM pigs

($P < 0.0001$). As a result, the overall ADG of SF-pCD pigs was significantly higher than FARM pigs ($P < 0.0001$).

On D26, 19 days after the initial vaccination, the number of IFN γ secreting PBMC trended higher in SF-pCD pigs compared to FARM pigs. On D40, 33 days after the initial and 14 days after the booster vaccination, SF-pCD pigs demonstrated significantly greater response than FARM pigs (Table 8.2). Similarly, SF-pCD pigs had significantly higher Mhyo serum IgG antibody levels than FARM pigs on D40 (Table 8.3).

RID assays confirmed the absence of detectable bovine IgG in FARM pigs and minimal (0.3 ± 0.04 mg/ml) porcine IgG in SF-pCD pigs in D1 sera (Figure 8.1). High concentrations of bovine IgG were present in D1 sera of SF-pCD pigs and decayed rapidly over time, with a calculated half-life of 6 days. Similarly, porcine IgG was present in abundance (35.7 ± 7.5 mg/ml) in D1 sera of FARM pigs and decayed over time but remained above 4 mg/ml for the duration of the study. Porcine IgG in SF-pCD pigs began to rise at D13 and gradually increased to approximately 3 mg/ml on D40 (Figure 8.1). The D40 serum porcine IgG concentration of FARM pigs was significantly higher than that of SF-pCD pigs (Table 8.3).

8.4. Discussion

This study yielded some unexpected results. The fact that the pre-weaning growth performance of SF-pCD pigs did not differ to that of FARM pigs is noteworthy (Table 8.1). SF-pCD pigs were bottle-fed for a short period (usually less than 48 hours) before they learned to drink from a liquid feeder from which they were fed four times per day. This frequency is drastically different than conventionally reared pigs which typically suckle more than 20 times per day.¹⁶⁰

Accordingly, it was a concern whether SF-pCD pigs would be capable of achieving growth rates

consistent with pigs raised on commercial farms. However, the results described here demonstrate that this goal is achievable using the established management protocol of SF-pCD pigs.⁷⁷ Further, the ADG of SF-pCD pigs surpassed that of FARM pigs after weaning, which is likely because SF-pCD pigs had less competition for food than their on-farm siblings (SF-pCD pigs: 2 pigs per pen with one feeder in each pen; FARM pigs: 6 or 7 per pen with one feeder in each pen).

The reason that cellular and humoral immune responses to Mhyo vaccination were quantitatively higher in SF-pCD pigs than FARM pigs in this experiment is not fully understood, however, differences in the intestinal microbiota of the groups is a possible contributor. It is reasonable to speculate that the intestinal microbial community structures between the two groups in this experiment are substantially different due to the different environments and pre-weaning diets. There is evidence in human medicine indicating that the neonatal intestinal microbial community regulates systemic immunity.¹¹ Further, pigs raised in environments with different hygiene levels and with different intestinal microbial compositions were shown to exhibit different mucosal immunity characteristics.⁸¹ It is not clear whether these factors may have affected the systemic immune responses of pigs used in this experiment. Results of this experiment however, suggest that environmental factors affect the systemic immune response. Whether or not this is due to differences in the intestinal microbiota is of interest but beyond the scope of the current study.

Another possible explanation for the stronger immune responses in SF-pCD pigs is that FARM pigs experienced higher postweaning stress. It is well known that pigs experience considerable stress shortly after weaning, demonstrated by a delay of weight gain and an increase in serum cortisol concentration.⁵⁴ Increased serum cortisol is considered to be immunosuppressive. It is reasonable to speculate that SF-pCD pigs in this experiment experienced less postweaning stress

because pen density and feeding competition was lower than experienced by FARM pigs.

Although this may potentially explain the stronger immune responses observed in this study, it should be noted that the increased cortisol levels in postweaning farm pigs are reported to return to normal within 6 days,⁵⁴ and the timing of vaccinations in this experiment (D7 and D26 of age), were beyond this postweaning stress period. Thus, if postweaning stress contributed significantly to the findings in the current study, the period of postweaning stress is likely to have extended beyond 6 days postweaning in the FARM pigs.

Differences in the composition of the suckling (milk) diets may have also contributed to the observed differences in the immune response between SF-pCD and FARM pigs. SF-pCD pigs were mainly fed bovine colostrum while FARM pigs suckled sow colostrum and milk before weaning. A large number of bioactive substances had been identified in bovine colostrum, such as immunoglobulin, insulin-like growth factor (IGF)-I and -II, epidermal growth factor (EGF), and lactoferrin among others.¹⁵ Both bovine and porcine colostrum are rich in immunoglobulin. However, the mature bovine and porcine milk contains a much lower concentration of immunoglobulin.^{16,56} Thus, it is clear that SF-pCD pigs, when consuming bovine colostrum throughout the entire preweaning phase receive quantitatively more immunoglobulin than FARM pigs. Similarly, bovine and porcine colostrum contain similar levels of IGF-I, but the mature milks have about 10-fold less. Thus, SF-pCD pigs should also have received more IGF-I throughout the suckling phase. Thus, greater consumption of immunoglobulin and IGF-I may have contributed to the higher immune response of SF-pCD pigs to the Mhyo vaccine relative to FARM pigs.

The differences in immune responses observed in this study need not discourage the use SF-pCD for research purposes. Although the intestinal microbial community composition of SF-pCD pigs

is likely different than that of FARM pigs, the fact that SF-pCD pigs are colonized by bacteria is probably more important. It has been demonstrated that gnotobiotic pigs which are not colonized by any bacteria, do respond to certain antigens, and thus have impaired immune function.¹⁸ In light of this phenomenon, SF-pCD pigs should process similar immunity to conventional pigs in a qualitative sense. It is also unknown whether the magnitude of the difference in immune response observed between groups in this study is biologically relevant, i.e. that one group would be more or less susceptible to Mhyo challenge. Interestingly, it has been reported that increased serum Mhyo antibody titers but decreased IFN- γ production by PBMC were associated with superior protection against Mhyo challenge. (SF-pCD pigs had higher IFN- γ response and antibody titers).¹⁸⁵

In conclusion, SF-pCD pigs had superior growth performance and higher cellular and humoral immune responses to Mhyo vaccination compared to their siblings reared commercially using industry standard procedures. The fact that SF-pCD pigs possess qualitatively similar immune response as FARM pigs suggests that SF-pCD pigs have immune functions that resemble those of conventional pigs, and are thus suitable for swine infectious disease research.

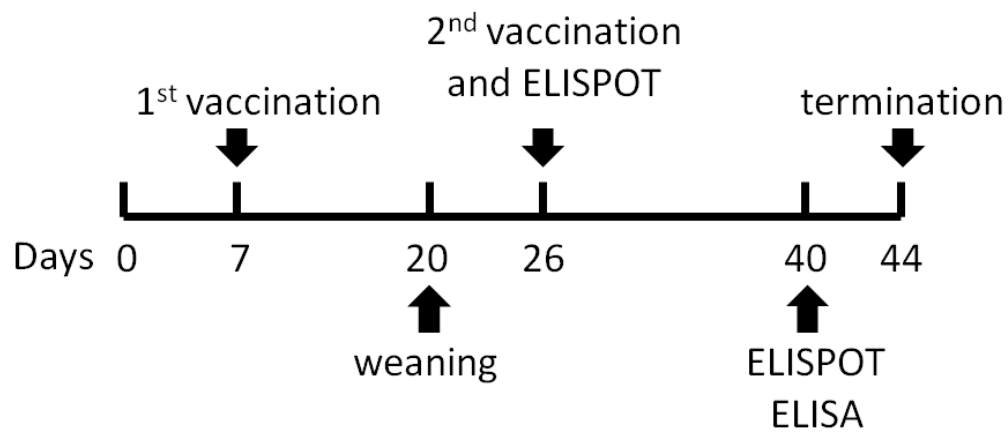


Figure 8.1. Experimental design. The times (days of age) of major events the experiment are noted in this figure.

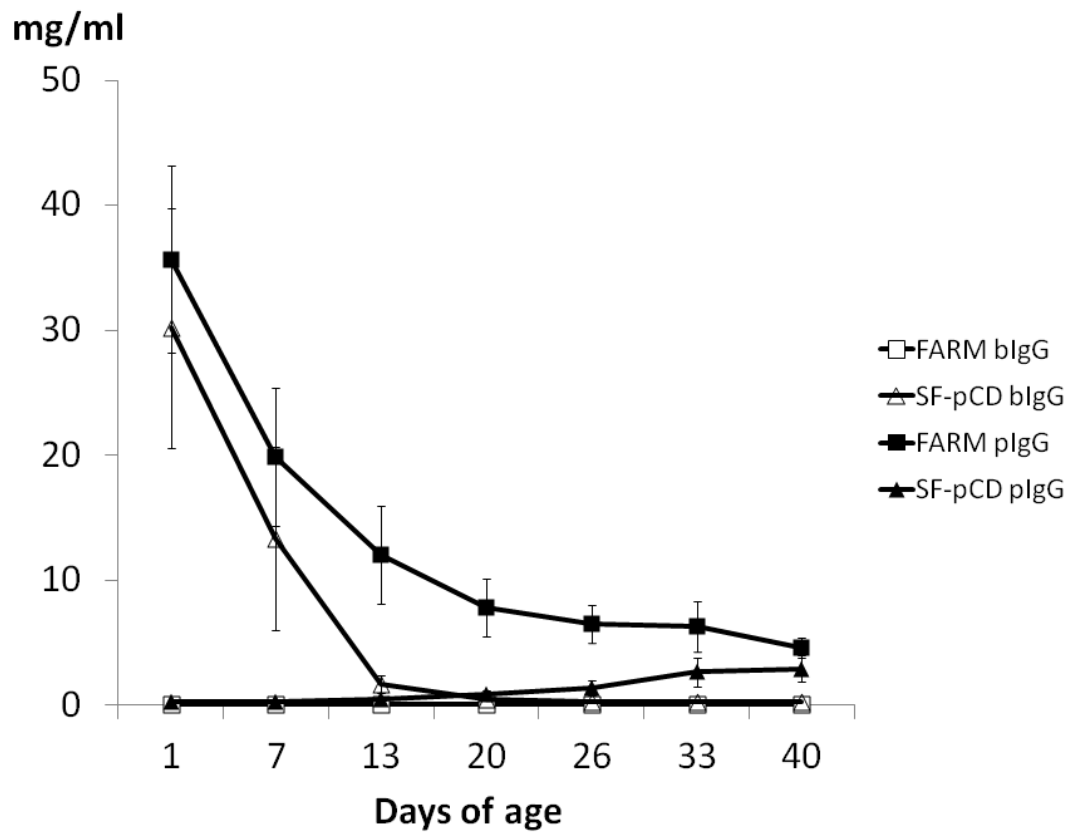


Figure 8.2. Mean serum porcine and bovine IgG concentrations in SF-pCD (n=12) and FARM pigs (n=14). The vertical bars represent standard deviations.

Table 8.1. Body weights and average daily gains of SF-pCD and FARM pigs

	SF-pCD (n=12) (kg)		FARM (n=13) (kg)		<i>P</i> value
	Mean	SD	Mean	SD	
Body weights					
D1	1.45	0.28	1.39	0.19	ns
D20	6.58	0.70	6.71	0.88	ns
D40	17.14	1.57	12.45	1.77	<0.0001
Average daily gains					
Pre-weaning*	0.27	0.03	0.28	0.05	>0.05
Post-weaning*	0.53	0.05	0.29	0.08	<0.0001
Overall	0.40	0.04	0.28	0.04	<0.0001

All pigs were weaned 20 days of age.

Table 8.2. Numbers of IFN- γ -secreting PBMC in response to *Mycoplasma hyopneumoniae* antigen stimulation in SF-pCD and FARM pigs

	SF-pCD (n=11)		FARM (n=12)		<i>P</i> value
	(#/10 ⁶ cells)		(#/10 ⁶ cells)		
	Mean	SD	Mean	SD	
D26	578.98	205.70	314.30	275.03	0.07
D40	684.69	360.13	312.23	255.64	<0.0001

Table 8.3. Day 40 serum *Mycoplasma hyopneumoniae* titers and porcine IgG concentration in SF-pCD and FARM pigs

	SF-pCD (n=12)		FARM (n=13)		<i>P</i> value
	Mean	SD	Mean	SD	
<i>M.hyo</i> titers	447.9	208.3	336.8	290.5	0.03
Porcine IgG (mg/ml)	2.9	1.2	4.6	0.8	0.05

-
9. Attempted experimental reproduction of porcine periweaning-failure-to-thrive syndrome using tissue homogenates

The results from Chapter 7 and 8 showed that SF-pCD is a suitable model for swine infectious disease research. Finally, in Chapter 9, an attempt was made to reproduce PFTS in SF-pCD pigs by inoculation of tissue homogenates from PFTS pigs. This experiment is important for the final conclusion as to whether PFTS is an infectious disease.

This Chapter has been submitted for publication. The copyright of this Chapter will belong to the journal in which it is published.

Huang, Y and J. Harding. Attempted experimental reproduction of porcine periweaning-failure-to-thrive syndrome using tissue homogenates. PLOS ONE, 2013. Submitted, under revision.

Huang, with Harding's assistance, designed and performed the experiment, analyzed the data and contributed to manuscript writing.

9.1. Introduction

Porcine periweaning failure-to-thrive syndrome (PFTS) is typified by newly weaned pigs, apparently healthy at weaning and without residual sickness from the suckling phase, that develop anorexia, lethargy and progressive debilitation within a week after weaning. The crude flow prevalence of PFTS in North America was recently estimated to be approximately 4%.¹³⁸ A portion of affected pigs show repetitive oral behaviour such as chomping and licking, which is regarded as an important characteristic of PFTS.⁷⁹ The most frequent lesions of diagnostic relevance are superficial gastritis, small intestinal villous atrophy and thymic atrophy, all of which are observed with higher odds in PFTS-affected versus non-affected animals⁷⁶ (and Chapter 5). The etiology of PFTS is unknown. To date, common porcine pathogens, specifically type 2 porcine circovirus (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus, transmissible gastroenteritis virus (TGEV) and *Mycoplasma hyopneumoniae* have been shown conclusively not to be associated with PFTS^{76,154} (and Chapter 5), whereas haemagglutinating encephalomyelitis virus (HEV), porcine enterovirus CPE groups 1, 2 and 3, rotavirus groups A, B and C, porcine enteric calicivirus (PECV), porcine cytomegalovirus (PCMV) and coccidia (likely *Cystoisospora suis*) may be detected in PFTS pigs but detection is not consistent across cases, and the presence is not associated with clinical status^{76,154} (and Chapter 5).

A critical step in testing the hypothesis that PFTS is an infectious disease is to determine if it can be transmitted to susceptible animals from affected animals. The ability to conduct these experiments is dependent upon having a reliable animal model based on experimental pigs that are immunologically naïve to the presumptive infectious agent(s). Commonly used models for studying swine diseases include the specific pathogen free (SPF), caesarean-derived

colostrum-deprived (CDCD) and gnotobiotic models. Each model has advantages and limitations which have been previously reviewed.⁷⁷ We have previously developed a snatch-farrowed, porcine colostrum-deprived (SF-pCD) pig model for infectious disease research.⁷⁷ SF-pCD pigs experience the advantages of natural birth, are raised on bovine colostrum until weaning at 20 days, can be inoculated during the suckling phase and are raised in conventional biocontainment level 2 (BCL2) facilities. Further, SF-pCD pigs are able to mount immune responses similar to conventional pigs but are free of maternally derived antibodies to diseases endemic to the source farm (Chapter 8).

The objective of this study was to determine if the clinical signs of PFTS, specifically repetitive oral behaviour and progressive loss of weight and body condition, could be reproduced by inoculating SF-pCD pigs with tissue homogenates derived from pooled organs of PFTS-affected pigs.

9.2. Materials and Methods

9.2.1. Ethics statement

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20110059).

9.2.2 Experimental design

Twelve SF-pCD pigs were born at the Prairie Swine Centre Inc. (Saskatoon, SK, Canada), and raised at the animal care unit (ACU) at the University of Saskatchewan (Saskatoon, SK, Canada) as described previously.⁷⁷ Briefly, the pigs were snatched-farrowed, disinfected and placed in

HEPA-filtered containers without contacting any farm equipment. Upon arrival at the ACU (day (D)0), pigs were bottle fed for 1-2 days, then transitioned as soon as possible to self-feeders until weaning at D21. For the first 21 days, a liquid diet consisting mainly of bovine colostrum was fed. At D21, all pigs were weaned on to an appropriately formulated dry starter diet free of all swine by-products including spray-dried porcine plasma.

On D14, piglets were conveniently allocated to two inoculated (INOC1, n=4; INOC2, n=4) and two control (CTRL1, n=2; CTRL2, n=2) groups. The control groups were relocated to a separate isolation room and appropriate biosecurity measures implemented to prevent cross contamination between rooms. A 20% w/v tissue homogenate consisting of equal amounts of tonsil, brain, lung, spleen, stomach, small and large intestines collected from 3 PFTS-affected pigs⁷⁶ was prepared in minimum essential media (MEM, Life technologies Inc. Burlington, ON, Canada). The tissues had been stored for 8 months at -80°C and thawed immediately before preparation of the homogenate. The homogenate was prepared fresh on each day of inoculation. On D14, INOC1 received 20 ml of the tissue homogenate orally via a gastric tube, while INOC2 received the same dose of homogenate orally as well as 2 ml of 0.2 µm-filtered homogenate both intramuscularly (IM) and intraperitoneally (IP) (Table 9.1). CTRL1 received 20 ml MEM orally. CTRL2 received 20ml MEM orally, 2ml MEM IM and 2ml MEM IP. On D21, the IM and IP inoculations were re-administered to all pigs.

To determine the pathogens present in the inocula, a sample of the filtered homogenate was cultured on blood agar aerobically at 37°C overnight. The filtered and non-filtered homogenates were tested for PCV2⁵⁵, PRRSV (Tetracore EZ-PRRSV™ Kit; Rockville, MD, USA), influenza A virus,¹⁸⁴ group A rotavirus (Chapter 5), HEV,¹⁷⁹ TGE,²⁰¹ PEV 1, 2, and 3,²¹⁰ PCMV,⁶³ PECV,²⁰³ *Helicobacter-pylori*-like organism and *Helicobacter-heilmannii*-like organism²⁸ and *M.*

*hyopneumoniae*¹¹⁴ by PCR.

All piglets were monitored twice daily for any clinical signs including repetitive oral behaviour and weight loss. Rectal temperature and body weights were measured daily until D37 of age. Pre-inoculation sera were tested by PCR as previously described¹¹⁶ to confirm the absence of PCV2 viremia. Pre-inoculation PRRSV testing was not undertaken since the barn of origin was known to be negative.

Piglets were euthanized and necropsied when clinical signs progressed to the point where animal welfare was compromised. Routine aerobic and anaerobic bacterial cultures were performed by Prairie Diagnostic Services (PDS) Inc. (Saskatoon, SK, Canada) on appropriate samples from pigs that developed progressive dyspnea, fever, anorexia and lethargy. All remaining pigs, including controls, were euthanized on D49 (35 days after the first inoculation, 28 days after the second inoculation). For all pigs, a necropsy was performed and multiple tissues collected for routine histological examination.

9.2.3 Statistical analyses

Body weights were compared on D14 (day of first inoculation), D29 (15 days after first and 8 days after second inoculation) and D49 (termination) using Mann Whitney's U test. Linear regression models were used to compare average daily gain (ADG) from D14 to 29 (ADG 14-29), ADG 30-49 and ADG 14-49, while accounting for body weight at the start of the period. All final models were checked for linearity, normality and homoscedasticity of residuals. Pigs euthanized prior to D49 (n=3) were excluded from the weight and ADG analyses. Fever-days (total days of a pig with rectal temperature $\geq 40^{\circ}\text{C}$) and diarrhea-days (total days of a pig having diarrhea) from D14 to 29 of age were compared between groups by Mann Whitney's U test.

Since there were no obvious differences in the frequency of fever and diarrhea, or in body weights between INOC1 and INOC2, CTRL1 and CTRL2, the two INOC groups and two CTRL groups were combined for statistical analyses. Further, because no significant clinical signs were observed after D29, the statistical analyses for fever and diarrhea were only performed on data from D14 to D29. All statistical analyses were performed using IBM SPSS Statistics version 21 (Armonk, NY, USA). *P* values less than 0.05 were regarded as statistically significant and values between 0.5 and 0.1 were considered indicative of a trend.

9.3 Results

The filtered and non-filtered homogenates tested negative for PCV2, TGE, influenza A virus, PRRSV, group A rotavirus, PECV, *M. hyopneumoniae* and *Helicobacter-pylori*-like organism, while positive for PCMV, PEV CPE groups 1, 2 and 3. *Helicobacter-heilmannii*-like organism was detected in the non-filtered but not the filtered homogenate. There was no bacterial growth from the filtered homogenate under aerobic conditions on blood agar.

All pigs grew at acceptable rates and appeared in good body condition before the first inoculation at D14. One pig in INOC1 (oral only) and two pigs in INOC2 (oral+IM+IP) developed progressive dyspnea, fever, anorexia and lethargy after the first inoculation and were humanely euthanized before the second inoculation. The first evidence of illness was observed on D15, D15 and D17 respectively in these three pigs, which were euthanized on D18, D16 and D20 respectively. Postmortem and histological examination revealed bronchopneumonia (2/3), pericarditis (3/3), pleuritis (3/3), and peritonitis (2/3) associated with mixed infections of lung, spleen and synovium with *E. coli*, *Streptococcus suis*, *Fusobacterium* spp. and *Staphylococcus* spp.. Two additional INOC1 and two INOC2 pigs developed transient fever of 1 to 2 days duration between the first and second inoculation but remained otherwise healthy. One pig in

each CTRL group developed fever for one day each, on D23 and D20 respectively, but also remained otherwise healthy. When all pigs, including those euthanized, were included in the analysis, the numbers of fever-days were not significantly different between INOC and CTRL (Table 9.2) during the 2 weeks period following the first inoculation. No INOC pigs developed illness or fever after the second inoculation.

Between D14 and D29, transient diarrhea characterized by small to moderate amounts of watery feces for 1 to 2 days developed in all but one INOC pig. Diarrhea was also observed in 3/4 CTRL pigs during this period. The numbers of diarrhea-days were not significantly different between INOC and CTRL (Table 9.2) during the 2 weeks period following the first inoculation. No diarrhea was noted following the second inoculation.

Body weights of surviving animals did not differ between groups on D14, D29 or D49 (Table 9.3). The ADG 14-29 of surviving pigs trended higher in CTRL than INOC, and ADG 29-49 was significantly higher in INOC than CTRL (Table 9.3). Body weight at D29 was positively related with ADG 29-49 ($P=0.001$, $\beta=0.044$ kg/d). ADG14_49 did not differ between groups (Table 9.3).

All pigs regardless of treatment showed repetitive chomping and licking behaviour typical of PFTS after weaning. This oral behaviour was noted for a brief period of time from 24 to 48 hours after weaning from liquid diet on D21, and before the pigs learned to eat dry starter diet from the feeder. The behaviour ceased abruptly as soon as solid feed was consumed.

9.4 Discussion

The current study represents the first attempt to experimentally reproduce PFTS using tissue homogenates from PFTS-affected pigs and the SF-pCD model. Although chomping was

observed in both groups, it is clear that the current approach failed to reproduce the progressive loss of weight and body condition within 2 weeks of weaning that is characteristic of the syndrome. Nevertheless, the results of this study provide important and novel insights.

The inoculation strategy of this experiment aimed to maximize the likelihood of reproducing PFTS. Firstly, two inoculations were performed: one week before and on the day of weaning. Though typical clinical signs of PFTS are by definition observed shortly after weaning, it is possible that if caused by infective agent(s), the initial exposure occurs during the suckling phase. For this reason, the first inoculation was performed before weaning. The second inoculation was on the weaning day, when pigs experience stresses associated with a change of diet and a reduction in oral immunoglobulin consumption. Secondly, the combination of different inoculation routes mimicked both gastrointestinal and systemic exposure. Although the most frequently observed lesions of PFTS suggest the gastrointestinal tract to be a primary organ associated with the pathogenesis (Chapters 4 and 5), it is also possible that a systemic infection causes anorexia that induces secondary lesions of lymphocytic gastritis and small intestinal villus atrophy. The application of oral, IM and IP inoculation was an attempt to reproduce all these possible paths to the pathogenesis of the disease. Thirdly, the combination of multiple tissues in the homogenate accounted for the possibility that infectious agent associated with the etiology of PFTS might reside in non-gastrointestinal tissues such as brain, lung and spleen. However, using multiple tissues in the inocula increased the risk of diluting a presumptive infectious agent if that agent was localized in some but not other tissues. With these uncertainties in mind, our inoculation strategy for this first attempt was designed to ensure early and broad exposure. Following the first inoculation it was clear that oral inoculation of non-filtered homogenate caused bacterial septicemia, which is not a feature of PFTS. It was for this reason that the second

inoculation used only filtered inoculum.

The failure to reproduce progressive loss of weight and body condition characteristic of PFTS in this experiment suggests the etiology of PFTS may not be infectious, although a definite conclusion cannot be made based on a single experiment. Although unlikely, it is also possible that the causative agent of PFTS (if any) in the inoculum was not in sufficient concentration to cause clinical sickness. Further, the non-filtered tissue homogenate was only inoculated into the pigs once, since after the first oral inoculation of non-filtered tissue homogenate, some pigs developed septicemia and the others not affected by septicemia failed to develop progressive loss of weight and body condition. However, this approach potentially weakened the possibility of demonstrating a bacterial etiology.

It was clear that the clinical signs in the three INOC pigs euthanized following first inoculation were associated with septicemia of mixed bacterial origin, demonstrating that the SF-pCD pigs were susceptible to “opportunistic” bacterial pathogens. The polyserositis however, observed in these three 3 INOC pigs was not consistent with PFTS (polyserositis is not a primary lesion of PFTS).

A number of other potential pig pathogens were retrospectively identified in the inoculum including PCMV, PEV and *Helicobacter-heilmannii*-like organism. In spite of this, no histological findings consistent with *Helicobacter-heilmannii*-like organism (also known as *Candidatus Helicobacter suis*),⁶⁸ PEV²¹⁰ or PCMV were observed indicating these potential pathogens failed to induce characteristic lesions or disease in this experimental model. In agreement with other studies⁷⁶ (and Chapter 5), these data provide additional evidence that these organisms are not the cause of PFTS.

Most INOC pigs developed diarrhea and fever between D14 and D29. However, diarrhea and fever was also observed in some CTRL pigs that remained otherwise healthy, and the diarrhea-days and fever-days were not significantly different between INOC and CTRL pigs (Table 9.2). In our experience, pre-weaning diarrhea is frequently observed in SF-pCD pigs and although the mechanism is not fully understood, the diarrhea resolves soon after weaning. The diarrhea in CTRL therefore was not unexpected, and the diarrhea observed in the INOC pigs may be a combination of “physiological”, nutritional and pathological diarrhea.

An interesting finding in this experiment was that repetitive oral behaviour (chewing and chomping) was observed in all pigs (INOC and CTRL) shortly after weaning and before the pigs ate solid feed. Although the presence of excessive, repetitive oral behaviour is clearly associated with PFTS, chomping was not induced by inoculation in this study. This observation led to the suspicion that chomping may be a behaviour associated with hunger or abdominal discomfort. Indeed, when sows are feed restricted, repetitive sham chewing is a well recognized stereotypic behaviour.¹⁰² Moreover, our group has also documented repetitive oral behaviour in a small proportion of commercial nursery pigs 1 and 4 weeks post weaning in the absence of any obvious disease (unpublished data). Collectively, these findings indicate that the repetitive oral behaviour is not specific to PFTS.

A recent experiment conducted at the University of Minnesota attempted to reproduce PFTS by inoculating pigs with HEV, group A rotavirus, or a combination of HEV, group A rotavirus and PRRSV. It was reported that clinical signs of PFTS were observed in all inoculation groups as well as in a sham-control group. Unfortunately, the observed clinical signs were not specified, nor did body weights differ among groups.¹⁹⁸ It is obvious that that experiment also did not reproduce progressive loss of weight and body condition, which is an important feature of PFTS

and a fundamental part of the clinical case definition.⁷⁹ Further, no histological changes characteristic of PFTS⁷⁶ were observed in the experiment.¹⁹⁸

The current experiment also serves to verify that the SF-pCD pig is a valid model for study of infectious diseases. Although specific pathogen free (SPF), Caesarian-derived, colostrum-deprived (CDCD) and gnotobiotic pig models are commonly used for swine infectious disease studies, these models have disadvantages. SPF pigs are typically conventional pigs that have diminished levels of maternal antibodies after weaning. Despite its convenience and economical nature, one cannot use younger SPF pigs due to the high levels of maternal antibodies. Thus, this model is not suitable to study the effect of pathogens on suckling pigs, especially when the pathogen of interest is prevalent making it difficult to locate a seronegative farm. CDCD and gnotobiotic pigs do not experience natural birth. It is well documented that before natural birth, pigs and other livestock species experience a pre-parturient cortisol surge¹⁷⁴ that is important for tissue maturation, immunoglobulin absorption and glycogen deposition in muscle and liver.⁴⁹ This may explain why Caesarean-derived pigs typically have higher mortality than naturally delivered pigs even if delivered at term. An additional drawback of gnotobiotic pigs is that they exhibit a distorted immune response because they lack bacterial colonization in the gut.¹⁸ This indicates the gnotobiotic model is not always a satisfactory model although it has undoubtedly served as a powerful tool for swine infectious disease research in the past.

The development of the SF-pCD model addresses the weaknesses of other swine models. Previous efforts to raise SF-pCD pigs by other researchers resulted in survival rates of 80% or less.^{12,141} After some modification, we have consistently raised (non-inoculated) SF-pCD pigs with 100% survival.⁷⁷ Further, it has been shown that SF-pCD pigs were able to mount an immune response similar to that of conventional pigs (Chapter 8). The use of SF-pCD pigs in this

present experiment, despite the failure of reproducing the body weight loss associated with PFTS, demonstrates that these pigs are susceptible to systemic bacterial infection. This study thus provides additional verification that SF-pCD is a valid model for infectious disease research. Although susceptibility to viral pathogens has not yet been demonstrated, SF-pCD pigs are presumably susceptible.

In conclusion, the progressive loss of weight and body condition, a key feature of PFTS and part of the clinical case definition, was not reproduced by inoculating SF-pCD pigs with tissue homogenates from PFTS-affected pigs. This study therefore provides further evidence that PFTS is not caused by an infectious etiology.

Table 9.1. Treatment groups and inoculation schedule for PFTS inoculation study

Groups	Day 14		Day 21	
	Oral	IM+IP	Oral	IM+IP
INOC1 (n=4)	20 ml non-filtered	NA	NA	2 ml filtered each route
INOC2 (n=4)	20 ml non-filtered	2 ml filtered each route	NA	2 ml filtered each route
CTRL1 (n=2)	20 ml MEM	NA	NA	2 ml MEM each route
CTRL2 (n=2)	20 ml MEM	2ml MEM each route	NA	2ml MEM each route

IM = intramuscularly, IP= intraperitoneally, MEM = minimum essential media

Table 9.2. Number of days with diarrhea or fever during the two week period following first inoculation (D15 to D29)

	INOC	CTRL	<i>P</i>*
	(n=8)	(n=4)	
Diarrhea	15/88	4/60	0.368
Fever ($\geq 40^{\circ}\text{C}$)	8/87	2/60	0.368

* *P* values of Mann Whitney's U test.

Table 9.3. Median body weight (kg) and average daily gain (ADG; kg/d) at selected time points following inoculation at day 14 and 21

	INOC* (IQR)†	CTRL (IQR)	<i>P</i>
Weight D14 (pre-inoculation)	3.1 (1.4)	2.5 (1.1)	0.683
Weight D29 (14d post inoculation 1)	5.8 (2.2)	5.6 (3.0)	1
Weight D49 (termination)	15.5 (4.3)	13.6 (5.8)	0.556
ADG D14 to D29	0.17 (0.07)	0.20 (0.13)	0.1
ADG D29 to D49	0.49 (0.10)	0.40 (0.16)	0.03
ADG D14 to D49	0.35 (0.06)	0.32 (0.13)	1

*INOC, n=5; CTRL, n=4; Euthanized (septicemic) pigs were excluded from analyses

† IQR = Interquartile range

10. General discussion, conclusions and future directions

This section does not aim to restate the discussions of previous research chapters, but to consider the findings of all the chapters and discuss points that have not been sufficiently addressed in the discussion section of each chapter. Finally, several overall conclusions of the research will be drawn and future directions for investigation of PFTS suggested.

Although Chapter 2, which has been published in the *Journal of Swine Health and Production*⁷⁹, was not based on a well-controlled experimental study, it was foundational to the investigation presented in this thesis since it established a clinical definition of PFTS, without which the subsequent studies were not possible. It should be emphasized that the case definition is at a clinical level, and thus may not be highly specific and sensitive. The definition was the beginning, not the result, of the investigation so future modification based on scientific evidence is anticipated. Further, proposing PFTS as a clinical syndrome does not imply that the author is committed to there being one single, common etiology. It was the clinical similarities that justified grouping all of the herd outbreaks of “starve-out” pigs together and the assignment of a name that facilitated the investigation.

As it turned out, the investigation demonstrated that similarities in gross, histopathological and serum parameter observations, between affected and unaffected pigs, were largely consistent among pigs and farms (Chapter 4, 5 and 6). These findings are important, not because they served to elucidate an infectious etiology (with exception of the superficial gastritis, see discussion below), but that they demonstrate further similarities between PFTS-affected pigs and farms, which provide evidence that these farms and pigs were affected by the same clinical syndrome or pathogenesis. The changes of histology and clinical pathology were strongly

associated with PFTS, which satisfied the first of Hill's epidemiological criteria for causation (i.e. the strength of the association).⁷³ Further, the consistency of the association among farms satisfied Hill's second epidemiologic criterion.⁷³ It should be clear that there are different levels of causation. The infectious organism causes histological lesions in an infectious disease, and the lesions cause the clinical expression of the disease. Thus, these lesions may remain in the causal chain of PFTS and are useful to investigators for the development of etiological hypotheses. The screening for rotavirus A in chapters 4 and 5, for example, was largely driven by the presence of villous atrophy. On the other hand, as discussed in chapters 4 and 5, in a case-control study, the inability to demonstrate events in temporal sequence (Evan's fourth criteria⁴⁶) makes it impossible to know whether the histological changes were the cause or the result of the clinical disease. Further, except for the superficial gastritis, the other highly prevalent lesions (small villous atrophy and thymic atrophy) lack specificity to PFTS, and could be caused by any etiology that induces anorexia or reduced feed intake. This does not fulfill Hill's third criterion.⁷³ As discussed in chapter 5, it is possible that the superficial gastritis observed in PFTS pigs was a direct result of anorexia or starvation. This is based on literature demonstrating that in mice and rats, gastric erosions could be induced by starvation. However, it is also notable that the current evidence relating gastric lesions with starvation cannot be directly extrapolated to conclude that the gastritis observed in PFTS pigs is caused by anorexia or starvation. Thus, it is important to investigate the etiology of the gastritis, which, if not caused by starvation alone, may be the key lesion that may elucidate the etiology of PFTS.

Although an etiology was not identified in this research, significant progress was made that will assist in the diagnosis of PFTS. As demonstrated in Chapter 5, the lack of at least two of the three lesions, namely, thymic atrophy, superficial gastritis and small intestinal villous atrophy,

can be used to rule out PFTS with confidence. Obtaining a positive diagnosis of PFTS is more complex and requires the fulfillment of the clinical case definition, presence of characteristic lesions, and the absence of other known pathogens and associated lesions that can explain the clinical signs. Because the prevalence of PFTS is thought to be low, it is more likely for a diagnostic lab to receive a submission that is actually not PFTS. Thus, having a simple and confident rule out criterion at the histological level is helpful.

In this study, the search for pathogens was mostly targeted at viruses. Although the author is justified not intensively searching for bacterial pathogens because of the absence of indicative lesions, this is one limitation of the current study. The major lesions observed in PFTS pigs lack neutrophilic infiltration, a hallmark of bacterial disease. However, not all bacteria cause neutrophilic reaction, with *Lawsonia intracellularis* which cause hyperplasia of intestinal crypts being a good example. Thus, although not likely, the author cannot conclude with great certainty that PFTS is not associated with one or more less characterized bacteria.

The exact weaning age and its association with PFTS is another factor that could not be investigated in this study. It is understandable that in the production system, that all pigs weaned on the same day were not born the same day. Thus, the same batch of nursery pigs may have an age variation of up to 7 days. If early weaning is a risk factor of postweaning starve-outs, and if one batch of nursery pig contains many that were prematurely weaned, an “outbreak” of starve-out (hence PFTS) may occur and then appear to wane in subsequent weeks as the number of prematurely weaned pigs decreases.

The age of the onset is another important factor that needs to be considered when investigating an etiology for PFTS. Does PFTS begin at weaning, or does it begin sometime during the

suckling phase? The results presented in this thesis alone cannot answer this question. However, an incidental observation from one control pig in Chapter 8 sheds some light on the temporal origin of PFTS. This pig suckled its biological dam on farm, but was noticed to be lethargic, anorexic, thin and continually lost weight after weaning. The pig was excluded from the experiment, and was euthanized 13 days after weaning. Interestingly, the only gross and histological changes for this pig were thymic atrophy, superficial gastritis and small intestinal villous atrophy. No further diagnostics were performed on this pig. This pig's condition was consistent with those of a postweaning starve-out. On review of this pig's body weight record, it was interesting to note that it was growing above the average of other sibling piglets until it experienced a growth arrest approximately one week before weaning. Taken together, these observations support the possibility that the onset of PFTS may be before weaning. If true, the PFTS-affected pigs selected for diagnostic workup in the current investigation may have been in a chronic stage, which could reduce the chance of successfully identifying the infectious organisms involved in the initiation of the disease process.

Chapters 7 and 8 described the development of the SF-pCD pig model in preparation for the inoculation study presented in Chapter 9. These works are significant because they demonstrated that SF-pCD pigs represent those raised in conventional farms, so that the future experimental results generated from SF-pCD pigs should mimic those in the field situation. Further, the development of SF-pCD pigs is a significant contribution to swine infectious disease research. The advantages of SF-pCD pig had been well discussed in these chapters and those discussions will not be reproduced here.

Chapter 9 showed that inoculation of tissue homogenates from PFTS pigs into SF-pCD pigs failed to reproduce PFTS. This is an indication that PFTS is a non-infectious disease, and it

agreed with the findings that no pathogens were identified as the etiology of PFTS from the investigation in Chapters 4 and 5. However, it should be clear that the absence of evidence is not (at least not always) evidence of absence. The author is fully aware of potential weakness in the inoculation study presented in Chapter 9. The dose of the inoculation was not evaluated nor optimized, even though the quantity of a putative infectious organism in the inoculum is understandably a very important factor for successful reproduction of the disease. The same applies when considering the inoculation route. Additionally, the tissues for the inoculum were stored in -80°C for about 8 months before the study. This may further lower or even inactivate the potential infectiousness of the organisms in the inoculum. Finally, the SF-pCD pigs consume large volumes of bovine colostrum, which contain large amounts of immunoglobulin. If there is a cross-protective immunoglobulin in the diet, it will potentially protect against the pathogen and prevent the reproduction of PFTS even if it is an infectious disease. Thus, it is overstated to conclude that PFTS is not an infectious disease based on the available evidence, but this is certainly less likely based on the results of this research. Moreover, the failure to reproduce PFTS in Chapter 9 discouraged the further pursuit of additional inoculation trials using tissue homogenates. It is the author's opinion that efforts put into identifying potential infectious organisms by molecular methods would be more rewarding. If a putative organism is discovered, one should attempt to isolate and culture the organism and then perform inoculation studies using pure culture.

All together, the author concludes that:

1. PFTS is a clinical syndrome with consistent pathological and serum analytical changes in affected pigs.

2. There is a lack of evidence that PFTS is an infectious disease. Based on the efforts of this research to establish an infectious etiology, this lack of evidence swings the pendulum in favour of PFTS being a non-infectious disease.

11. Future directions

The investigation of PFTS is not completed. The author suggests some future directions for the investigation.

Firstly, a fasting trial should be performed. A common suggestion the author received from conference audience and manuscript reviewers was that PFTS is simply the result of pigs that failed to “learn to eat” after weaning (i.e. PFTS is the same as “starve-outs”). And it might be that if pigs are fasted for a certain period of time, they will enter a non-reversible state such that reintroduction of feed is not possible. The author is inclined to agree with this suggestion, but solid evidence should be obtained by experimentation. A fasting and re-feeding trial of newly weaned pigs is a powerful way to answer this question (Will prolonged fasting result in a non-reversible feed refusal state that leads to PFTS?). It was the author’s observation that even with feed placed in front of PFTS pigs, they were not interested in the feed. Thus, if in a fasting trial, fasted pigs readily eat solid feed after re-feeding, it will suggest that fasting alone does not lead to PFTS, but other factors must be involved in the pathogenesis (i.e. it was not because the PFTS pigs did not find the feed, but something makes them not want to eat). Further, if fasted pigs do not have the same lesions (especially superficial gastritis) as identified in PFTS pigs, an additional etiology must be sought to explain the presence of these characteristic lesions.

Secondly, a more detailed investigation targeting suckling pigs on PFTS-affected farms should be performed. As discussed above, if the initiation of PFTS begins in the suckling phase, sampling only weaned pigs may reduce the chance of identifying the etiology, regardless of whether it is infectious or non-infectious.

Thirdly, a prospective study in farms affected with PFTS can be helpful to identify individual risk factors associated with PFTS, such as weaning age. Cortisol levels could also be measured in such a study to elucidate whether PFTS is associated with increase level of stress.

Fourthly, more powerful techniques to reveal novel infectious organism should be employed. Indeed, high throughput sequencing of stomachs and brains from PFTS pigs is underway. The author is looking forward to obtaining these results as they may provide additional evidence of whether or not PFTS has an infectious etiology.

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